# PART I

*Myosin, Actin, and Actomyosin*

# INTRODUCTION

Like most children, the biochemist, when he finds a toy, usually pulls it to pieces, and he can seldom keep his promise to put it together again. The loveliest toy ever provided by nature for the biochemist is the contractile muscle fibril. No wonder that biochemists of the fame of Danilewsky, Halliburton and v. Fürth tried to pull it to pieces as early as the eighties. They were fairly successful, showing that a considerable part of the fibril could be dissolved by strong salt solutions. A globulin-like protein was obtained which readily precipitated on dilution and was called "myosin" to distinguish it from the water-soluble "myogen."

Interest began to center around "myosin" again in the third decade of our century when H. H. Weber and, somewhat later, J. T. Edsall and A. v. Muralt took up its detailed study.

When my laboratory started work on muscle some seven years ago, our first step was to pull "myosin" to pieces. The earlier "myosin" turned out to be an undefined mixture of two proteins which united to form a compound. The properties of the pure components were quite different from those of "myosin" itself. For instance, they were quite soluble in water, whereas the most characteristic feature of "myosin" was its water-insolubility.

One of these new proteins will still be called "myosin." In retaining this name I wanted to pay tribute to the pioneers of this field. The other protein, discovered by F. B. Straub [16], will be called "actin."

Neither of these two proteins is, in itself, contractile. If put together in proper relations they unite to form the complex "actomyosin," which has the remarkable property of contractility. Contraction can be induced *in vitro* by ATP and ion constituents of the muscle fibre.

To see actomyosin contract was one of the greatest im-

pressions of my scientific career. Motion is one of the most basic biological phenomena, and has always been looked upon as the index of life. Now we could produce it *in vitro* with constituents of the cell.

Before embarking on the analysis of this phenomenon, I should like to make a general remark. Most of the properties and reactions of myosin, actin, and actomyosin seem unique, if not paradoxical. The reason is simple. As you know, all basic biological phenomena use up energy. The system performing these basic biological functions must have two states, rest and activity, each characterized by a different content of free energy. The proteins performing these reactions can also be expected to have two different states, and can be expected to be fibrous and to make part of the water-insoluble structure of the cell. These proteins generally refuse to be extracted without denaturation. Students of proteins thus limited their attention to the soluble, easily extractable globular proteins, which perform rather secondary functions around this basic, insoluble structure. What have been described as characteristics of "protein" are but qualities of the easily extractable globular fraction.

Owing to the specific mechanical function of muscle, its particles are fitted together in such a way that they can be taken apart without serious damage. Myosin can be obtained even in crystalline condition rather easily, so that most of the experiments referred to in this book were performed with recrystallized material. All the same, I hesitate to call myosin a true "substance." It is a whole world, a living little organism which releases and uses up energy, takes up and gives off water, leaning on a less reactive particle, actin, to translate these changes into motion.

A new fibrous protein called "tropomyosin" has been described recently by K. Bailey (1946). Its quantity is rather small as compared to that of actin or myosin, and since there is no evidence that it is involved in the contractile mechanism, its discussion will be omitted.

## I. Myosin

### *General Properties*

Myosin is a hydrophilic colloid: it dissolves in water giving a limpid solution. Its IP is pH 5.2. In the presence of small concentrations of KCl it readily crystallizes at pH 6.5–6.7 in the form of fine needles, forming small bundles (Figs. 1 and 2).

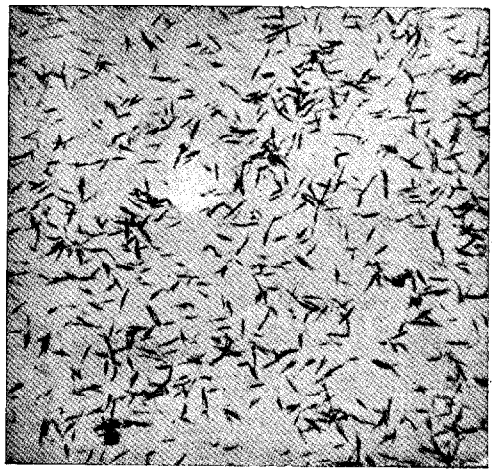


Fig. 1. Myosin crystals. Magn. 1:90.

Whether the crystals would satisfy the crystallographer, I do not know. I rather doubt it. Certainly they are beautiful enough to delight the heart of the biochemist. The first spinning experiments made with this material by Spellman and Erdös (personal communication) on the ultracentrifuge at Upsala show that it is perfectly homogeneous. The same is shown by the cataphoretic experiment of Erdös (personal communication).

On analysis, this recrystallized myosin showed the following composition:

| | | | |
|---|---|---|---|
| C | 50.04% | N | 16.15% |
| H | 7.70% | S | 1.14% |
| | Ash | 1.23% | |

According to this analysis, myosin is a protein. It contains six S atoms for every UW, and no P. The myosin contained 3% lipoidic matter, partly insoluble in acetone. This was removed prior to combustion. Although myosin behaves like a globulin

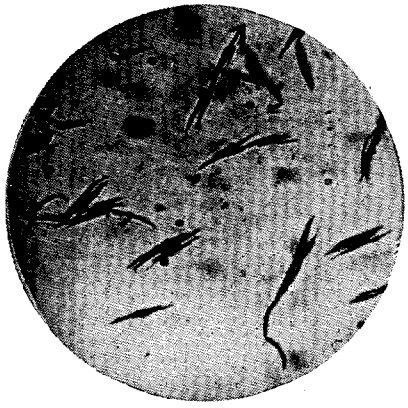


Fig. 2. Myosin crystals. Magn. 1:300.

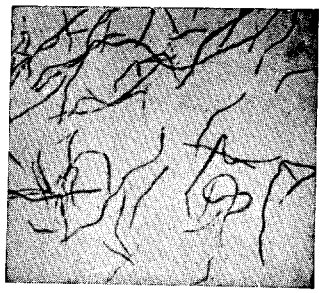


Fig. 3. Myosin with incipient acid denaturation. Magn. 1:90.

in that it precipitates with $(NH_4)_2SO_4$ as we pass half saturation, it cannot be classed with the globulins because it is soluble in water.

Myosin is very unstable, though not all of its properties are equally sensitive. It is completely denatured by acids or anhydrous solvents. Even at pH 7 its viscosity rises and becomes anomalous if it is incubated at 37° for ten minutes, or if it is stored for a long time at 0°. Salts and ATP, which increase its hydration, have a stabilizing influence. Slightly denatured myosin has a tendency to form threads (Fig. 3). Native, actin-free myosin readily spreads on water. On drying — even in the frozen state — myosin becomes insoluble.

If dissolved in salt-free water, myosin gives a limpid and rather viscous solution with a splendid DRF. Neither the high viscosity nor the DRF, however, are expressions of a fibrous

character, but are due to association, or swarm formation. If salt is added in higher concentration, or if the pH is raised, the viscosity drops and the DRF disappears. Myosin in many ways resembles soaps which, at certain pH's, also display a splendid DRF and high viscosity owing to association of the particles.

Myosin has a unique property: though hydrophilic, it is quantitatively precipitated from its watery solution by very small concentrations of neutral salts, like KCl. Such a small concentration as 0.001 M salt is sufficient to cause turbidity, and 0.025 M KCl causes complete precipitation. This reaction

TABLE I. *The Precipitating Action of Salts on Myosin*

| | 0.2 | 0.1 | 0.5 | 0.025 | 0.0125 | 0.006 | 0.003 | 0.0015 | 0.0008 | 0.0004 |
|---|---|---|---|---|---|---|---|---|---|---|
| KCl | 0 | 0 | + | ++ | ++ | ++ | + | + | 0 | 0 |
| KF | + | + | + | ++ | ++ | ++ | ++ | ++ | + | 0 |
| KJ | 0 | 0 | + | ++ | ++ | + | 0 | 0 | 0 | 0 |
| LiCl | 0 | 0 | + | + | ++ | ++ | ++ | + | + | 0 |
| NaCl | 0 | + | + | ++ | ++ | ++ | + | + | 0 | 0 |
| $MgCl_2$ | 0 | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + |
| $CaCl_2$ | 0 | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + |

0.5 ml of the salt solution was added to 2 ml of a 0.1% salt-free myosin solution. Upper line: final molar concentration of the salt. 0 means no change, + means turbidity or precipitation.

is not specific for KCl and is shared by other neutral salts (Table I). If the salt concentration is increased the precipitate dissolves again.

For crystallization one generally starts with myosin dissolved in strong salt solution and precipitates the protein by dilution. A 5% myosin, dissolved in 0.5 M KCl, is somewhat opalescent, has a relatively low viscosity, and shows no DRF. If the salt is gradually diluted by the addition of water to about 0.3 M, the solution becomes more opalescent and begins to display DRF, and its viscosity rises as the result of gradual aggregation, but no particles can yet be seen under the microscope. On stirring, a silky sheen appears, indicating the forma-

tion of elongated particles and regular distribution. When the KCl concentration has been decreased to 0.05 M, visible particles appear in the form of long, fine, needle-shaped crystals. Crystallization occurs at the height of this gradual association of particles and the gradual decrease of intermicellar distances. These distances, however, must be rather large even in crystals because the crystalline mass, if separated on the centrifuge, always includes great quantities of water (95%) only a portion of which can be located between the crystals; hence a considerable part of the water must be located inside of the crystals. Crystallization is greatly promoted by stirring which provides the necessary coaxial orientation of particles. In many ways the properties of myosin also resemble those of vegetable viruses as revealed by the study of Bernal and Fankuchen (1941).

Naturally, association and consequently the DRF are functions of concentration. A certain concentration of myosin in salt solution will show DRF, whereas a more dilute solution may not.

The viscosity of myosin, dissolved in 0.5 M KCl, is low as compared to fibrous colloids but high as compared to globular proteins (Fig. 4). The viscosity of fresh myosin is normal even

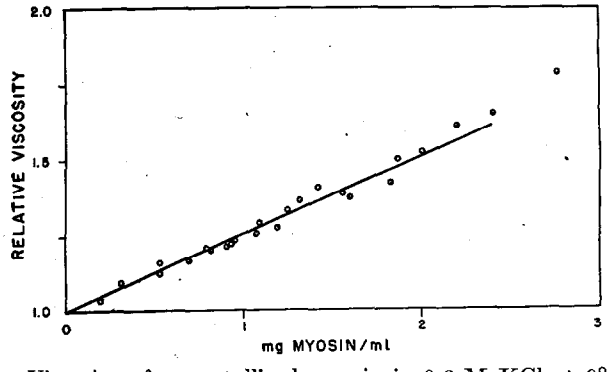



Fig. 4. Viscosity of recrystallized myosin in 0.6 M KCl at 0° (pH 7).

at pressures as low as 1 cm. water-pressure (Pittoni, unpublished). The viscosity seems to remain normal even at very high concentration, where the protein forms a soft, plastic gel.

All this indicates moderately elongated particles with a low degree of axial asymmetry.

According to the diffusion measurements of Weber and Stöver (1933) the MW of "myosin" is $10^6$ g., the length of particles 500 Å, and their width 45 Å (maximum values, Weber, 1939). On treatment with strong urea the MW dropped to $10^5$. As found by F. Guba (oral communication) the viscosity of such urea-treated myosin is as low as to indicate a globular shape, which suggests that the myosin micel is built of smaller globular units. Evidence will be given later, suggesting that units of MW of 17,000 and 150,000 are actually involved in the structure of the myosin micel. Unfortunately the measurements of Weber and Stöver, as well as later measurements of MW and molecular dimensions were made with impure myosin, more or less heavily contaminated with actin. More data are urgently needed.

### *Metal Myosinates*

If a myosin solution is precipitated by KCl and the precipitate separated on the centrifuge, treated with alcohol, dried and analyzed, it will be found to contain no Cl but rather large quantities of K. The discharge and precipitation of myosin is thus due to the unequal adsorption of the anion and cation present. The absence of Cl shows that above the IP myosin has no affinity for anions, while the relatively great quantities of K show a strong affinity for this cation. The action of K is not specific: as shown by T. Erdös [47] the affinity of myosin is the same for K and Na. If preference is given to K throughout my discussion, it is because K is the main cation of muscle and is estimated with greater ease than is Na. The high adsorption power of "myosin" towards K was noted first by C. Montigel (1943) in Verzar's laboratory.

The adsorption of K to myosin has been studied more in detail by I. Banga [00]. The result of one of her typical experiments is reproduced in Fig. 5 (upper curve). This curve shows that myosin binds K strongly. The main feature of this curve is that it is composed of two parts of different gradient. Both parts are, on a logarithmic scale, linear, corresponding thus to an

exponential function, as adsorption processes usually do. The two parts meet at 0.025 M KCl, where the myosin has adsorbed five equivalents of K per UW, is isoelectric, and is maximally precipitated. We should thus distinguish between two processes of adsorption: (1) the adsorption of the five first equivalents of $K^+$ by the negative myosin, and (2) the further adsorption of $K^+$ by the electro-neutral K myosinate. Let us call the first flat right-hand side of the adsorption, taking place at the lower K ion concentration, primary adsorption, and call the second, steeper left-hand side, corresponding to the higher K ion concentration, secondary adsorption. The fifth valency, making the system isoelectric, lying at the breaking-point of the curve, may be counted with either zone of adsorption.

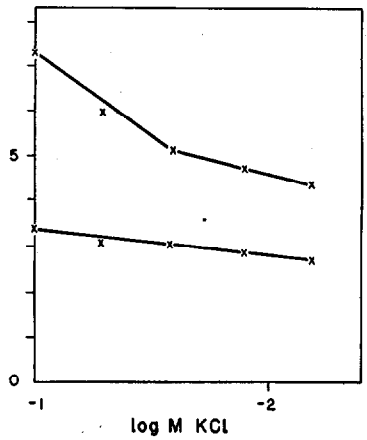



Fig. 5

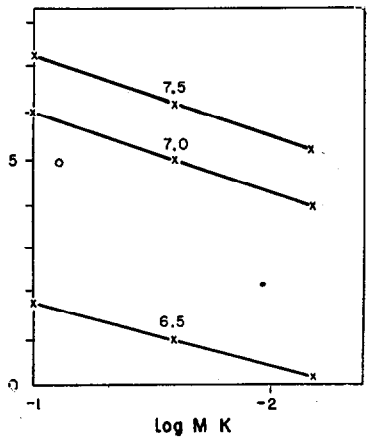



Fig. 6

Fig. 5. The adsorption of K on myosin in presence of KCl. Upper curve: fresh myosin 24 hours after the death of the animal. Lower curve: the same myosin 48 hours later. The ordinate shows the equivalents of K bound per UW of myosin.

Fig. 6. K-fixation of myosin at varied pH. Abscissa: log M KCl. Ordinate: equivalents of K bound per UW of myosin.

In the primary adsorption the basic negative charge of the protein prevails and the adsorption of K is supported by an electrostatic attraction which means that the $K^+$ is bound strongly, as expressed in the relative flatness of the curve; in

the secondary adsorption — as indicated by the steepness of the curve — the K is held loosely and $K^+$ is more readily taken up or given off on changes of K ion concentration in the surrounding fluid: a given change in the K ion concentration in the fluid will cause twice as much to be taken up or given off in this zone as in the primary one.

TABLE II. *Precipitation and Crystallization of Myosin*

| $\frac{Na_2HPO_4}{KH_2PO_4}$ | 0.1 | 0.05 | 0.025 | 0.0125 | 0.006 | 0.003 | 0.0015 | 0.0008 | pH |
|---|---|---|---|---|---|---|---|---|---|
| 1/0 | 0 | 0 | 0 | 0 | + | + | + | 0 | |
| 16/1 | 0 | 0 | 0 | ++ | ++ | + | + | 0 | 8 |
| 8/1 | 0 | 0 | 0 | ++ | ++ | + | + | 0 | 7.7 |
| 4/1 | 0 | 0 | + | ++ | ++ | + | + | 0 | 7.3 |
| 2/1 | 0 | 0 | +* | ++ | ++ | + | + | 0 | 7 |
| 1/1 | 0 | 0 | ++** | +++ | +++ | + | + | 0 | 6.7 |
| 1/2 | 0 | +** | ++** | ++* | ++ | + | 0 | 0 | 6.4 |
| 1/4 | + | + | ++ | ++ | ++ | + | 0 | 0 | 6.1 |
| 1/8 | + | + | + | + | + | + | 0 | 0 | 5.8 |
| 1/16 | + | + | + | + | + | + | + | 0 | 5.5 |
| 0/1 | + | + | + | + | + | + | + | 0 | |

Isomolar $Na_2HPO_4$ and $KH_2PO_4$ were mixed in different proportions (Column 1). The final molar concentrations of $PO_4$ are given in the upper line: 0 = no change, + = precipitation. Asterisks mean crystallization.

K ion adsorption is a very labile property of the myosin. Even storage for a few hours makes an appreciable difference. The secondary adsorption suffers first and the curve flattens out. The curves in Fig. 6 are taken with such a myosin stored for 24 hours. On storage for 48 hours both processes suffer. The lower curve in Fig. 5 is taken with the same myosin as the top curve 48 hours later.

To come back for an instant to Fig. 6, it can be seen that myosin, in the presence of 0.025 M KCl (which precipitates it completely) is discharged and isoelectric, with one $K^+$ adsorbed at pH 6.5. Myosin, at this pH, seems to have one negative charge, compared to five negative charges at pH 7. Table II shows that crystallization of myosin is limited to a very narrow

range and has its maximum at pH 6.7, with probably two negative charges neutralized by two $K^+$. Table II and Fig. 6 also explain why different authors, working in the presence of salts (buffers), found the IP of myosin anywhere between 5.1–6.6. As pointed out by Hollwede and Weber, the IP of myosin is 5.1–5.3, whereas the IP's of alkali-myosinates are higher.

The real IP of myosin is thus at pH 5.2 but in the presence of 0.025 M KCl the myosin will be isoelectric up to pH 7. Thus KCl extends the IP of myosin. The extension of the IP of proteins by neutral salts is a well known phenomenon. In fact, my very first biochemical paper, written under the guidance of L. Michaelis (1920) twenty-six years ago, describes the shift of the IP of casein. The extension of the IP of casein under the influence of neutral salts, however, is exceedingly small, compared to that observed with myosin. While the behavior of myosin is thus not specific qualitatively and represents a basic reaction of proteins, it is quite specific quantitatively. This very high adsorption power towards $K^+$, and cations in general, seems to be intimately connected with the function of myosin. As shown, however, by A. Lajta (see page 86) the structural proteins of kidney and brain have a similar affinity to K. This specific adsorption power seems to be one of the basic properties of structural proteins, performing basic biological functions, and seems to be intimately connected with the very nature of life.

The curve of Fig. 5 could not be extended any further towards the larger or smaller K concentration for technical reasons. The K adsorption could be measured only if the myosinate was insoluble and could be separated on the centrifuge. Above 0.1 or below 0.006 M KCl, the myosin dissolves (pH 7). We can thus correlate the physical state of myosin with the number of $K^+$ bound: with three $K^+$ and two negative charges left, myosin is freely soluble; with four $K^+$ and one negative charge left, it is slightly soluble; when all negative charges are balanced by five $K^+$, myosin is insoluble; and it is soluble again when it has seven $K^+$ bound. Association ceases, DRF disappears, and viscosity reaches minimum with nine $K^+$, as extrapolated for 0.3 M KCl.

In Fig. 7 taken from Banga, the adsorption of Mg and Ca are compared. The curve shows that the adsorption of Ca and Mg is subjected to the same regularities as that of K and shows a rather pronounced break at the fifth equivalent adsorbed. Ca

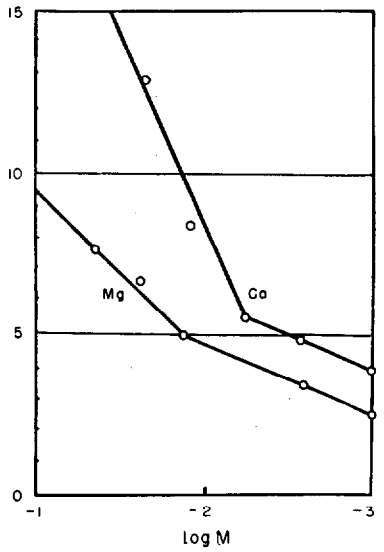



Fig. 7. Binding of Ca and Mg by myosin. Abscissa: log M concentration of the metal (as chloride). Ordinate: equivalents of metal bound per UW of myosin.

has the greatest affinity for myosin, which is demonstrated by the fact that the lowest concentration is needed from this ion to have five equivalents bound. Then Mg follows, and K is last. Therefore, if we have myosin in an isomolar solution of Ca and Mg, or Ca and K, the Ca will be adsorbed predominantly. Mg is preferentially adsorbed in an isomolar solution of Mg and K. (This was verified in the experiments of Banga.) The situation will be different if K is added in excess. If, for instance, myosin is brought into equilibrium with 0.12 M $MgCl_2$, from which five equivalents are bound, and KCl is added in 0.1 M concentration, two $K^+$ will be bound because 0.1 M KCl corresponds to the adsorption of seven equivalents, five of which are occupied by the Mg.

### *Myosin and Water*

As has been shown in the previous chapters, the colloidal behavior of myosin is governed by its metal adsorption. Like other colloids, the myosin particle has at the alkaline side of its IP a negative charge due to the dissociation of its acidic groups. The hydrophilic nature of myosin is evidently due to this negative charge. As the outside action of this charge is balanced more and more by adsorbed K ions, intermicellar distances decrease and the quantity of intermicellar water becomes less and less. The minimum is reached when the number of positive charges equals that of the negative ones, at pH 7 in 0.025 M KCl with five $K^+$ adsorbed per UW. At this point myosin is quite insoluble. This water-insolubility shows that the myosin particle has no inherent "hydrophility" comparable to that of, say, glycerol, which is due to the electropolar nature of its OH groups. This is in agreement with results of Hill and Kupalow who showed that out of the 800 g. of water contained in 1000 g. of frog muscle, not more than 30 g., i.e., 4%, can be bound.

If the KCl concentration is increased above 0.025 M, more K is adsorbed, and intermicellar distances increase again. With two adsorbed $K^+$ in excess, myosin dissolves again but is still strongly associated, as shown by the high viscosity, strong DRF, and silky sheen. On further increase of charge, association gradually ceases, viscosity reaches a minimum, and DRF disappears. This shows that the colloidal behavior and "hydrophility" of myosin are governed mainly by electric forces, and the water bound by myosin is mainly intermicellar water filling the space between micels which mutually repel each other. The high water-content of myosin crystals or precipitates shows that in isoelectric myosin the electric forces are poorly balanced and prevent the very near approach of different particles or the approach of different parts of the same particle. The concentration of water around the charged structure may also play a role in these short-range relations.

### *ATP Myosinate*

As mentioned before, myosin strongly binds metals but leaves (at neutral reaction) anions unbound. This property

holds even for trivalent anions, like ATP*, but occurs for metal-free ATP only. As K is adsorbed, myosin becomes more and more capable of binding ATP as well. As shown by Fig. 8, borrowed from W. Sz. Hermann [45], adsorption of K and adsorption of ATP are parallel. (The ATP-adsorption-curve in Fig. 8 could not be extended further to the left towards O $K^+$

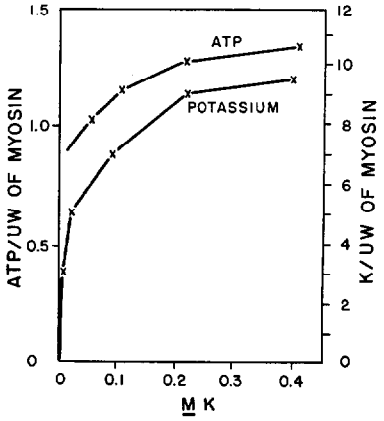



Fig. 8

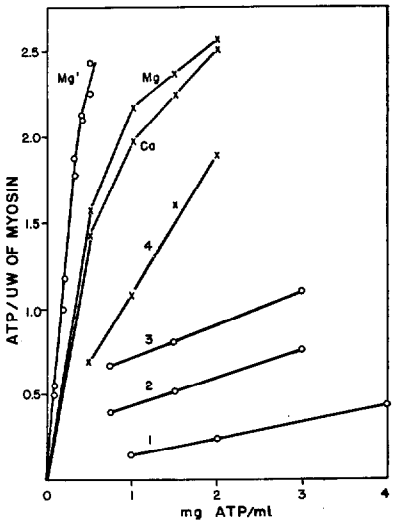



Fig. 9

Fig. 8. Adsorption of ATP and K by myosin. Left ordinate (relating to upper curve): molecules of ATP adsorbed per UW of myosin. Right ordinate: number of K adsorbed. Abscissa: [K] in the fluid. 0.17% ATP.

Fig. 9. Adsorption of ATP by myosin. Abscissa: ATP-concentration in the fluid. Ordinate: number of ATP-molecules adsorbed per UW of myosin.

because ATP, at neutral reaction, is a salt, containing in our experiments K as cation.)

The ATP adsorption is an exceedingly labile function of myosin and deteriorates quickly in storage. Curve 1 of Fig. 9 is an adsorption curve of ATP (Banga) taken at a time when no special care was exercised to use fresh preparations and no

*ATP is tetravalent but as shown by Banga the pK of the fourth acidic group is 8 and consequently, at neutral reaction, this group is not dissociated and ATP can be regarded as trivalent.

extra KCl was added. The curves 2 and 3 are corresponding curves of W. Sz. Hermann [45] using 24-hour-old myosin without and with, respectively, the addition of 0.2 M KCl. Curve 4 was taken by Hermann with myosin two hours after the death of the animal in the presence of 0.2 M KCl. This curve is much steeper than the previous ones, but all the same the adsorption at lower ATP concentrations is rather weak.

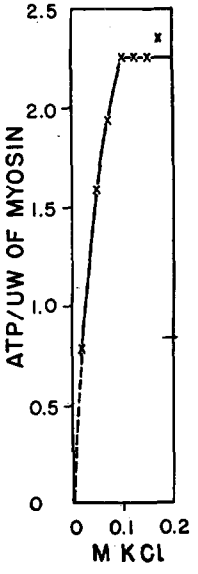



Fig. 10

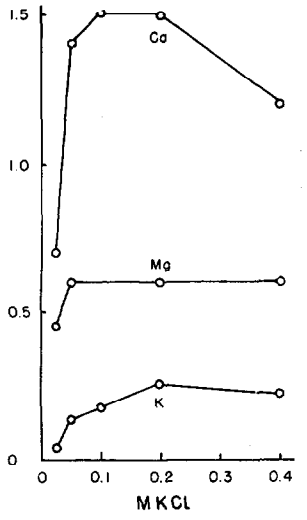



Fig. 11

Fig. 10. Adsorption of ATP in presence of 0.0005 M $CaCl_2$ and varied concentrations of KCl (abscissa). Ordinate: mols of ATP adsorbed by the UW of myosin in presence of 0.02% ATP. The broken line is extrapolated to O K.

Fig. 11. Adsorption of ATP at varied KCl concentrations in presence of 0.001 M $CaCl_2$, 0.001 M $MgCl_2$, and in the presence of K alone. Ordinate: molecules of ATP bound per UW of myosin. 0.2 mg ATP per ml.

Ca or Mg alone, in low concentration, does not cause ATP to be adsorbed. These ions cannot be tested in higher concentration because they precipitate ATP; in low concentrations (0.0005–0.001 M) they have no effect whatever on the ATP adsorption [Hermann, 45]. If added in the presence of KCl,

they very greatly increase the adsorption of the nucleotide. The Ca and Mg, adsorbed in the primary circle, change the general properties of the protein in such a way that it will intensely adsorb ATP if it has first adsorbed K ions, as shown by the curves Ca, Mg, and Mg of Fig. 9.

This rather striking effect of Ca and Mg is brought out more clearly if the KCl concentration is varied at constant Ca or Mg, and at a very low ATP concentration, such as 0.02%, as is the case in Fig. 10 [Hermann, 45]. The curve is exceedingly steep and reaches maximum critically at 0.05–0.1 M KCl. In spite of the low ATP concentration, much nucleotide is bound and the curve would have remained still straighter up to its maximum had not the ATP concentration in the fluid been reduced by the adsorption.

In these experiments performed by Hermann in the winter of 1945–46, Ca and Mg had the same effect. When these experiments were repeated in the spring of 1946, Ca showed the same effect as before but Mg was less active (Fig. 11). Whether the difference was due to seasonal variation or to methodical differences I am unable to state at present. It is not impossible either that in the earlier experiments a small quantity of Ca was retained by the myosin, careful purification being impossible in these very rapid preparations.

As shown before, myosin in 0.1 M KCl has two positive charges in excess. One would thus expect precipitation to occur on addition and adsorption of the negatively charged ATP. Instead, the opposite happens: ATP has a solvent action. If KCl is added to myosin in varied concentration with and without ATP, it will be observed that in the presence of ATP the precipitation is weaker, or no precipitate is formed at all, depending on the concentration of the ATP added. It can be deduced herefrom that the ATP adsorbed does not neutralize the positive charge of the adsorbed K ions.

### *Glycogen-Myosin Compounds*

If a watery solution of myosin is mixed with a solution of glycogen, a precipitate is formed. If, for instance, equal parts of 0.2% myosin and 1% glycogen are mixed, the protein is

carried down quantitatively. The resulting glycogen-myosin complex is not soluble in dilute (0.1 M) KCl. If, however, a small amount (0.05%) of ATP is added, the compound dissociates, the myosin and glycogen dissolve, and the solution now behaves as a solution of free myosin. Soluble starch or dextrin gives no precipitate with myosin under similar conditions. The glycogen compound is thus specific to some extent and behaves, in relation to ATP, analogously to the actin compound (see p. 17). It is possible that the formation of this glycogen-myosin complex and its reaction to ATP are involved in the mechanism and regulation of glycogenolysis, fixation and fermentation of glycogen being promoted by decreasing ATP concentration.

Polysaccharide compounds of "myosin" were first observed by Przyleczky, Majmin, and Filipovics (1934, 1935).

## II. Actin

The most interesting protein I have ever met is myosin; the second best is actin. The subtle reactivity of this latter and its interaction with ions are most fascinating objects of study. The actin particle is surrounded by a field of forces, governed by ionic balances, which forces determine the reactions of actin particles with one another and with myosin.

The most striking property of actin is its ability to exist in globular as well as in fibrous form. The properties of the two forms are widely different and so are the properties of the myosin compounds of both forms, the myosin compound of only the fibrous actin being contractile. The two forms can readily be transformed into each other reversibly, and this transformation from fibrous to globular and from globular back again to fibrous seems to occur in every contraction cycle.

The properties of actin are known — still rather incompletely — from the work of Straub and his collaborators, K. Balenović [17], G. Feuer and F. Molnár [62]. Actin is a protein and is readily denatured by heat. The preparation of actin, analyzed, showed the usual composition of proteins (51.3%C, 8.6% H, 15.1% N, 1.1% ash, and no P).

Unlike myosin, actin is not precipitated by alkali salts. It is less labile than myosin. Though it is readily denatured by salts below its IP, which is at pH 4.7, it can be precipitated at this pH, in the absence of salts, without considerable loss. It is readily destroyed by alkali or acid and is denatured by acetone but can be precipitated, under certain conditions, by strong alcohol or acetone in the native state. It is not precipitated by dilute (70%) alcohol.

Though present in resting muscle probably in the fibrous form, actin can be extracted from the tissue only in its globular form, and the method of extraction involves the change from the fibrous into the globular state.

Globular actin has all the earmarks of a typically globular

protein. Its viscosity is low and normal (Fig. 12); it has no DRF. On the subsequent pages it will be denoted by "G-actin," "G" standing for "globular."

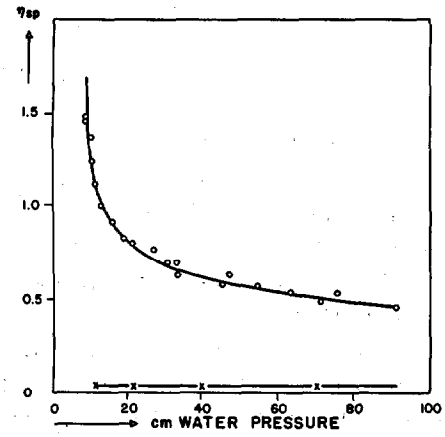



Fig. 12. Viscosity of globular and fibrous actin at various pressures. Crosses, 2 mg. globular actin per ml. in 0.2 M KCl. Circles, 3 mg. of fibrous actin per ml. in distilled water at 0° [Straub 16].

G-actin is freely soluble in water, giving a limpid solution. Its MW must be fairly low, 35,000–70,000 g. Its tryptophane content indicates that the MW is 68,000, but it passes readily through 10% collodion membranes which retain haemoglobin and let egg albumin through partially [62].

F-actin ("F" standing for "fibrous") has all the earmarks of a fibrous colloid. Its solution is opalescent and shows a strong Tyndall effect. This increase in light diffraction, as compared with that of G-actin, indicates that the formation of threads is not due to the unwinding of the globules of the G-actin but to their association in a row to something like a string of beads.

F-actin has a high and anomalous viscosity (Figs. 12 and 13). It shows a splendid DRF even on gentle shaking, which persists for some time after the fluid has come to rest (Fig. 14). It is fairly thixotropic, a property which contributes to the stabilization of the DRF. Stronger solutions set to a gel which liquefies on gentle shaking. Its DR is positive. It is completely retained by collodion filters.

Though thixotropy makes the exact measurement of viscosity impossible, it can be stated that the viscosity of F-actin is not too high; the range is the same as that of myosin; its axial asymmetry must therefore be in the same range too, and cannot be compared to the axial asymmetry of really fibrous, high-polymer colloids. The strong DRF and viscosity anomaly cannot be explained on the basis of long, thread-like form of particles, but must be due (as was the case with myosin) to association, or swarm formation, which is supported here by thixotropic forces. This conclusion is in agreement with the behavior of F-actin on drying. In the vacuum desiccator, actin dries down to a thin and hard sheet which can be pulverized and redissolved.

### *Metal Actinates*

Actin is precipitated by very low concentrations of Ca, which reaction reveals a high affinity for this cation. It will be precipitated completely by 0.002 M $CaCl_2$. Straub's experiments (in which he estimated the decrease in concentration of

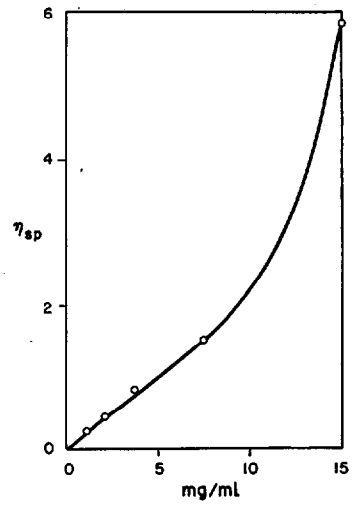



Fig. 13

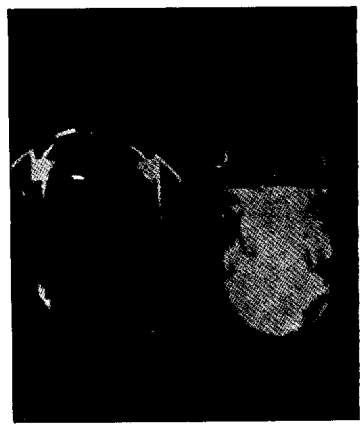


Fig. 14

Fig. 13. Specific viscosity of F actin as function of its concentration.
Fig. 14. Double refraction of actin solutions. Photographs taken through crossed nicols. Left: globular actin. Right: the same actin solution after polymerization to fibrous actin [Straub 16].

free Ca) show that the actin has bound six equivalents of the metal* per UW (pH 7). Erdos [57] studying the same phenomenon with Banga's somewhat modified method† (see "Metal Myosinates") found somewhat higher values (Fig. 15) and found no Cl in the precipitate. The curves obtained were

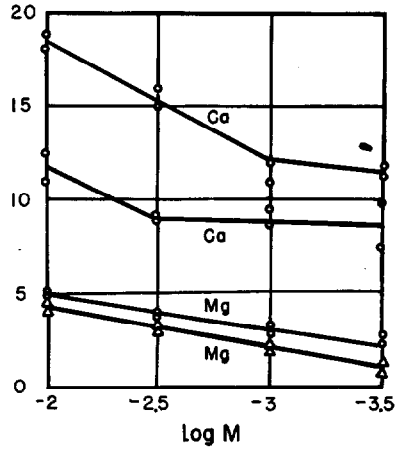



Fig. 15. Fixation of Ca and Mg by actin. Ordinate: equivalents of metal bound per UW of actin. Abscissa: log M Ca or Mg, present as chloride [Erdös 57].

analogous to the metal-binding curves of myosin in being composed of two straight parts and showing a break at the point where precipitation became maximal, indicating the complete "discharge" of particles. Accepting Straub's value, this happens with six equivalents of Ca per UW of actin as compared to the five equivalents found in myosin. The precipitation of actin by Ca salts is thus explained by the specific fixation of the metal and has the same mechanism as the precipitation of myosin. The precipitated Ca actinate can also be redissolved by an excess of the precipitating salt.

Actin is less readily precipitated by Mg which is explained by the lower affinity for this ion, borne out by the experiments of Erdös (Fig. 15). The affinity of actin for K and Na is very

*Ca fixation was found to have two optima, one at pH 7 and one at pH 9.

†Strong actin solutions were brought into equilibrium with different salt solutions through a cellophane membrane. After equilibrium was reached the actin was precipitated by acetone and the precipitate analyzed.

low and actin is not precipitated by these ions at all. The K bound in the experiments of Erdös was too low to be denoted in Fig. 15. In the presence of 0.1 M KCl, 2–2.5 K were bound, but no Cl.

There is a very fascinating antagonism between Ca and Na. If both ions are added to actin in the same relation as they are present in a physiologically "equilibrated" solution, as is Ringer's, no precipitate is formed at all, though Ca itself precipitates actin at these concentrations.

An explanation of this inhibitory effect is offered by Erdös [57] who found the fixation of Ca is inhibited by monovalent ions. The lower affinity of the latter may be compensated by their higher concentration. In the presence of 0.001 M $CaCl_2$ Erdös found ten equivalents of Ca bound in the absence of KCl, and three equivalents of Ca bound in the presence of 0.05 M KCl. This rather simple explanation probably does not reveal the whole situation, which will be shown later to be more complex.

The great affinity of actin for Ca is revealed also by the fact that actin preparations always contain one Ca per UW; so one Ca, at least, is bound very firmly. The relatively great affinity for Mg is revealed by the fact that actin preparations, obtained by Straub's original method, contain two Mg's per UW. Since both Ca and Mg are greatly diluted during the preparation, the conclusion seems to be warranted that these ions were not bound in the course of manipulation, but were linked to actin in the muscle, with their six equivalents, the probable number necessary for the complete neutralization of the outward charge of the particle. Since, in the course of preparation, the muscle is treated with strong KCl, the one Ca and two Mg's originally present seem not to be expelled by alkali-metals.

### *The G-F Transformation*

The polymerization of G-actin into F-actin is connected with a rise in viscosity which makes it possible to follow this process with ease in the viscosimeter. Any salt that does not denature actin will cause its polymerization. The monovalent cations are active in the order $Rb < K < Na < Li$; the anions, $J < Br < Cl < F$. Bivalent ions are much more active than

monovalent ones. The latter cause strong polymerization in 0.1 M concentration, the former in 0.005 M. The most active cation is $H^+$ and polymerization at pH 6 is instantaneous.

Mg has a quite specific effect. In the absence of Mg there seems to be no polymerization at all. If the rate of polymerization is measured in the presence of 0.1 M NaCl (pH 7.4) and varied concentrations of Mg, and the rate is coördinated with the Mg concentration (counting also the quantity of bound Mg) the curve, extrapolated to zero Mg, shows that in the entire absence of Mg there would be no polymerization at all. If the actin is incubated for half an hour with 0.001 M Calgon (sodium hexa-metaphosphate) which strongly binds Mg, the actin will not polymerize at all.* This action of Mg is quite specific and is not duplicated by Ca, and polymerization is not inhibited by oxalate (oral communication of Straub). Very small concentrations of Mg, like 0.0005 M, which in themselves have no polymerizing action at all, suffice to secure rapid transformation in the presence of 0.1 M NaCl. Mg sensitizes G-actin, or, more exactly, Mg actinate only is capable of polymerization.

There is a most fascinating relation between the action of different ions which shows close analogies to physiological equilibration. First of all there is an antagonism between Ca and Na. Ca in 0.005 M or NaCl in 0.1 M concentration induces rapid polymerization. If given simultaneously there is no polymerization at all: they completely balance each other.†

KCl is less active than NaCl in inducing polymerization of actin, but when such small concentrations as 0.002 M KCl are added to actin in the presence of 0.1 M NaCl, the polymerization is enhanced. These relations correspond to the relation of Na and K in Ringer's solution. This reaction shows that polymerization and the development of the forces surrounding the actin particle are not merely questions of charge or mutual replacement, but that other, subtler properties are involved.

*If Mg is added now in excess, actin readily polymerizes, showing that Calgon, as such, has no inhibitory action.

†Observations made at 0°. At room temperature, results are less reproducible. The Ca inhibition, observed in earlier experiments, must have been such an inhibition of the action of Na or K, because Ca, in itself, does not inhibit but is active.

The polymerization of actin is autocatalytic and its curve has the typical S-shape; it shows a more or less long latent period, then starts up suddenly, and proceeds at a very high rate to completion. The length of the latent period depends on the quantity of polymerized actin present at the beginning. The latent period is needed for the accumulation of the first effective quantity of F-actin. If the actin is stored for twenty-four hours in the ice-box before the experiment and time is given for the polymerized actin present to disaggregate, the incubation period becomes rather long and constant, but once the reaction starts it proceeds at the same rate as in fresh preparations. Ca, in inhibiting the polymerization in the presence of Na, acts by lengthening the latent period, and once polymerization starts, it proceeds at the usual high rate in spite of the presence of Ca. These details may have physiological importance and suggest that ionic balances cannot be looked upon as static environmental factors permanently conditioning certain kinetics. Their slight changes might have trigger actions or might be the safety buttons which block the trigger.

The rate of the G-F transformation increases greatly with the actin concentration and is also greatly catalyzed by myosin. In muscle, where the concentration of actin is high and myosin is present, polymerization may be exceedingly fast. The catalytic action of myosin has some rather puzzling features: 0.1 M KCl promotes the polymerization of actin and 0.6 M KCl does so even more strongly. Myosin, in 0.1 M KCl, strongly catalyzes the reaction but completely inhibits it in 0.6 M KCl, so that G-actin, which readily polymerizes in 0.6 M KCl, is perfectly stable in this solution if myosin is present. In order to make G-actin polymerize under these conditions the G-actomyosin formed has to be brought to dissociation, whereupon the polymerization readily occurs.

F-actin depolymerizes to G-actin if the ions are eliminated by dialysis at slightly alkaline reaction.

KI has a remarkable action: in 0.1 M concentration it induces polymerization, similarly to other alkali-salts, while in 0.6 M concentration it irreversibly depolymerizes F-actin.

## III. F-Actomyosin

If a dilute, watery solution of myosin and F-actin is mixed, the sudden rise of viscosity indicates the formation of a new substance. The viscosity is now not only high but also strongly anomalous (Fig. 16) and the solution displays a splendid DRF which is stronger than that of its components. If a more concentrated solution of actin and myosin is mixed, the liquid solidifies to an elastic gel. While myosin has an angle of isocline

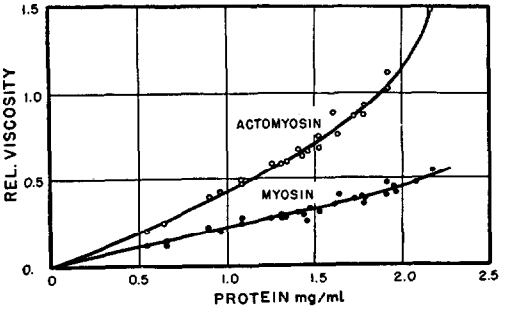



Fig. 16. Viscosity of actomyosin extracted from muscle. Upper curve: actomyosin. Lower curve: the same in presence of ATP. The latter curve is marked "myosin" since it is approximately identical with the curve of pure myosin. The actomyosin, in this case, was prepared from muscle as such and contained 1 part of actin to 5 parts of myosin.

approaching 78°, actomyosin, even at low velocities, readily orientates parallel to the wall (Mommaerts, 1945). All this indicates that out of two substances with relatively short particles a new substance has been formed, a typically *fibrous colloid with very long particles*. That this fibrous character is really due to the high axial asymmetry of the particles and not to some superficial association, is demonstrated by the fact that slight variation of pH or salt concentration does not affect these new properties. The substance, which is formed from actin and myosin, will be called "actomyosin."*

*Ardenne and Weber (1941) observed under the electron microscope very long threads in a "myosin" solution. These might have been actomyosin micels and were perhaps identical with the "heavy myosin" observed by Schramm and Weber (1942) in the ultracentrifuge. Similar threads were since observed by Hall, Jakus, and Schmitt (1946) in muscle extracts.

Actomyosin is not a stoichiometric compound; myosin and actin will unite to form actomyosin in all proportions, but the viscosity as well as other physical properties of these compounds will be different, depending on the relative concentration of the

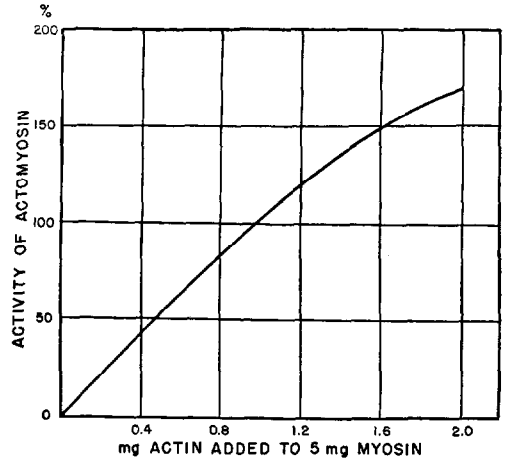



Fig. 17. Activity of F-actomyosin as function of the actin-myosin relation.

two proteins. The rise of viscosity, obtained on mixing actin and myosin, and the corresponding fall of viscosity obtained on dissociation of the compound have been termed "activity" in earlier papers and have been expressed numerically, 100% being

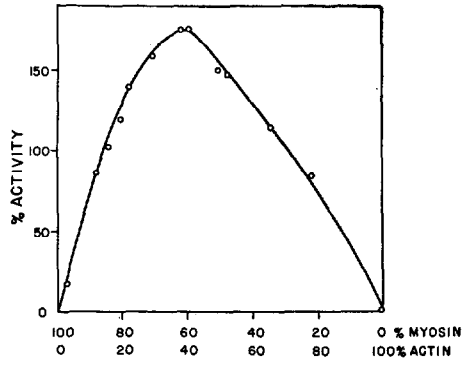



Fig. 18. Activity of F-actomyosin as function of the actin-myosin relation.

the viscosity of a certain standard preparation.* This expression of "activity" has since lost significance but was very useful at the beginning of this research as the first quantitative basis. Now it seems more adequate to express the composition of actomyosin with a ratio, giving the relative proportions of the two proteins.

Figs. 17 and 18, borrowed from Balenović and Straub [17], show the dependence of the "activity" on the actin: myosin ratio. The activity of the compound, at a given myosin concentration, increases at first proportionally to the concentration of actin, reaches a maximum, and then falls off again. The maximum is reached if the relative concentration of actin and myosin is 2:5. According to the measurements of Balenovic and Straub, muscle contains 2:5 or maximally "active" actomyosin. If not expressly stated otherwise, the term "actomyosin" will refer to such 2:5 actomyosin.

The colloidal properties of actomyosin are additive. Actomyosin is slightly thixotropic and exceedingly hydrophilic. Its solution is opalescent; the formation of actomyosin from its elements entails an increase of light diffraction.

Actin unites with myosin to form actomyosin only in the presence of small quantities of Mg (0.0005 M). In the entire absence of Mg, actomyosin is unstable and dissociates, but can be stabilized by the addition of this ion, which indicates that forces instrumental in linking actin to actin resemble those linking actin to myosin.

Actin, in many ways, modifies the enzymatic properties of myosin (see below). Under conditions in which Ca inhibits the polymerization of actin it neutralizes these influences.

### *Metal Actomyosinates*

If neutral actin and myosin solutions are mixed in the presence of KCl and phenolphthalein, a red color appears

*100% was called the activity of "myosin B," the actomyosin obtained by extracting muscle at 0° with Weber's alkaline KCl for 24–48 hours. This actomyosin always had the same specific viscosity and contained one part of actin to five parts of myosin. Five parts of myosin are capable of bringing into solution and protecting from destruction one part of actin. Both 1:5 and the double of this, 2:5, seem to correspond to a special stoichiometric relation of actin to myosin.

showing that the reaction has become alkaline (Banga, oral communication). This alkaline reaction is evidently due to the release of bound K. Forces engaged in the adsorption of K seem to be used now for the adsorption of actin. Accordingly, the metal-binding capacity of actomyosin is somewhat smaller than would correspond to its myosin content. The difference is not considerable and the K-adsorption curve of myosin in free and bound condition shows no major difference. We can expect thus that the colloidal reactions of myosin, due to K fixation,

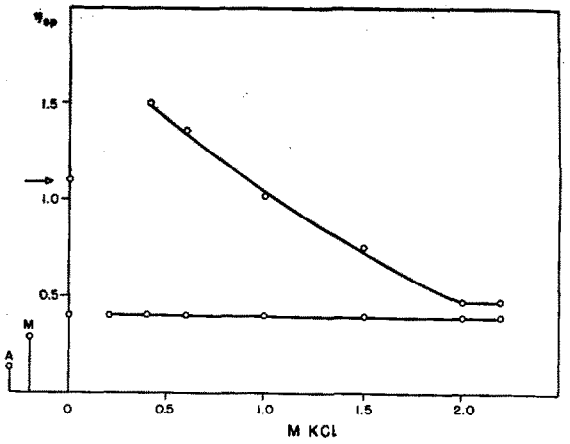



Fig. 19. Specific viscosity of actomyosin at varied KCl concentration in presence (lower curve) and absence of ATP (upper curve). 0.01 M veronal-acetate buffer of pH 7 in all samples, 0.015% ATP. The vertical lines on the left indicate the specific viscosity of the corresponding myosin and actin solutions. The viscosities of salt-free actomyosin with and without ATP (arrow) are marked on the ordinate [Guba, 58].

will be reflected in the reactions of actomyosin with the difference that a somewhat higher K ion concentration will be needed to produce maximal precipitation. This expectation is fully borne out by the experiment. The high hydrophility of metal-free myosin is reflected in the strong hydrophility of metal-free actomyosin, which causes extreme swelling of actomyosin gels in distilled water once their metal is completely washed out. This swelling can be prevented by 0.001 M KCl which also causes turbidity in free myosin. Increasing concentrations of KCl will cause more shrinking and turbidity

of actomyosin gels and increasing precipitation in actomyosin suspensions. Maximum of shrinking or precipitation will be reached (at pH 7) in the presence of 0.5 M KCl, as compared with the corresponding maximum of 0.025 M in free myosin. If the KCl concentration is further increased, shrinking or

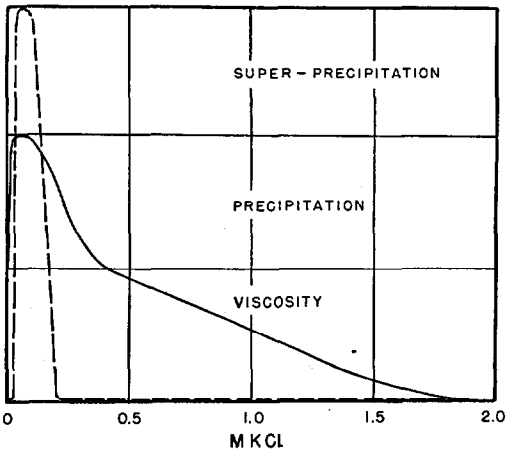



Fig. 20. Semiquantitative curve showing the behavior of actomyosin in presence (broken line) and absence (solid line) of ATP (0.1%) at varied KCl concentration.

precipitation becomes weaker again and 0.4 M KCl no longer precipitates; actomyosin remains dissolved and its viscosity can be measured. As the K ion concentration is further increased, the viscosity of the solution gradually drops, until at 2 M KCl it approaches asymptotically the additive value of actin and myosin, indicating the complete dissociation of the compound into the two free proteins. This is shown in the upper curve of Fig. 19. The solid-line curve, in Fig. 20, shows the whole course of events in a very rough, semi-quantitative way.

As can be expected from the properties of myosin, actomyosin, even at the maximum of its precipitation, is rather hydrophilic. Though it is precipitated completely, the precipitate includes rather great quantities of water which cannot all be located between the flocculi but must be enclosed within the particles. Accordingly, the shrinking of an actomyosin gel, caused by 0.05 M KCl, is but moderate. I have never succeeded

in preparing an actomyosin containing less than 95% water by precipitating the colloid with KCl and separating the precipitate on the centrifuge.

In a discussion about precipitating and redissolving actomyosin, one general remark cannot be suppressed. If actomyosin is precipitated (or its gel is brought to shrinking) cohesive links are soon formed between the particles, which makes redissolving increasingly difficult. The more the actomyosin is dehydrated, the sooner these links are formed and the stronger they are. In the case of maximum dehydration (obtained by ATP, as shown later) these cohesions develop within seconds.

Correlating the colloidal behavior with the curves of K fixation we may say, approximately, that actomyosin, with 3–9 $K^+$ bound by its myosin, is insoluble, remains in solution with more or less $K^+$ and dissociates completely with twelve $K^+$ bound (extrapolated). Dissociation of actomyosin thus depends on the charge of its myosin moiety; beyond a certain critical charge and hydration the myosin does not unite with actin. Actomyosin, on the whole, is more insoluble than free myosin and needs a higher charge to be dissolved. Like myosin, its colloidal behavior and hydrophilic character are governed by its charge.

Actin does not bind K; myosin does. So the K-fixation curve of actomyosin corresponds roughly to its myosin content. This does not hold for the case of Ca and Mg, these metals being bound by actin too. In this case the metal fixation is stronger than corresponds to the myosin content and the break in the curve is flattened out since the breaks of the myosin and actin curves do not overlap.

If actomyosin is dissolved in 0.5 M KCl and the salt is washed out by dialysis, the colloid solidifies to an elastic gel, provided its concentration is high enough (1–2%). This gelatinization of actomyosin solutions has been used by Weber (1933) in preparing his famous threads. He squirted his "myosin" solution in a thin jet into water. Since the salt was quickly washed out by the water, the actomyosin solidified in the form of a thin thread. Such threads have become classical objects of research. It is advisable to use a dilute (0.05 M) KCl

instead of water since, if the salt is washed out too thoroughly, the threads swell up enormously and become useless. The threads in 0.05 M KCl consist of actomyosin, maximally precipitated by KCl. They contain about the same quantity of water (about 98%) as the original solution, which shows their hydrophility. Actin-free myosin does not form such threads, so the "myosin" of Weber must have contained considerable quantities of actin. The threads, if kept for some time, become increasingly insoluble, even in M KCl.

One must be careful to use glass-distilled water, free of heavy metals. The heavy metals, usually present in common distilled water, are strongly bound by the threads which become denatured. Hereby they become elastic. While native actomyosin threads break when stretched by 10–15%, Cu-denatured threads may be stretched 300% which makes them suited for different physical studies but unsuited for the study of the subtler biological phenomena, like contraction.

### *Actomyosin and ATP*

Except for the formation of very long particles, actomyosin up to this point in the discussion has not revealed any reaction that could not have been predicted from the properties of its constituents. A highly specific and unexpected reaction occurs if ATP is added which, in the case of free myosin, had no other action than to increase solubility and hydration.

That ATP has a specific influence on the physical properties of "myosin" was demonstrated first by Engelhardt, Ljubimowa, and Meitina (1941) who showed that the extensibility of "myosin" threads is increased in a specific way by small concentrations of the nucleotide. J. Needham and his collaborators Shih-Chang-Shen, D. M. Needham, and Lawrence (1941) and later Kleinzeller, Miall, Dainty, D. M. Needham, and Lawrence (1941) found that DRF and viscosity of "myosin," dissolved in strong KCl, were decreased by small concentrations of ATP.

The action of ATP on actomyosin depends on the KCl concentration and is demonstrated, rather roughly, by the broken curve in Fig. 20. In this figure the abscissa corresponds to complete dissociation of the actomyosin into its

components. If we compare the two curves of actomyosin, obtained in the absence and the presence of ATP, we may say that ATP makes all reactions rather exaggerated and abrupt. Where KCl, in itself, caused maximal precipitation, ATP made this precipitation very much more intense. Outside this narrow range, ATP causes complete dissociation. What J. Needham and his collaborators have seen is evidently this dissociation.

Making the precipitation much more intense means that while KCl itself caused the actomyosin suspension to precipitate in the form of loose, voluminous flocculi, in the presence of ATP a granular precipitate is obtained which settles quickly to a small volume at the bottom of the test tube. If the ATP is added to the KCl precipitate, the loose flocculi change into a granular precipitate of small volume which indicates the extreme shrinking and dehydration of the particles. This precipitation, obtained in the presence of ATP, will be called "*super-precipitation*" to distinguish it from the simple salt precipitation obtained by KCl alone.

The visible effect of ATP depends on the physical state of the actomyosin. If this is present as an inhomogeneous suspension, super-precipitation will be observed; if it is present in the form of a continuous gel, the same action will express itself in an extreme degree of shrinking and dehydration. This shrinking is especially striking if the gel has the form of a thread (Fig. 21a). Owing to the relatively big surface, the ATP will diffuse into the gel quickly and so the shrinking will be very fast. For a few seconds nothing will happen, when the ATP pervades the gel; suddenly shrinking sets in, and within half a minute or so the thread shrinks to a small dense stick* (Fig. 21b). This shrinking is so fast that it gives the impression of active contraction and henceforth will be called "*contraction*" to distinguish it from the rather slow and moderate shrinking and dehydration caused by KCl alone.

*Sometimes the action is so violent that the outer layers contract before the interior can follow, with the consequence that the former break up, giving the appearance of a "crocodile skin" to our thread. This will more often be the case with actomyosin prepared from pure actin and myosin. The thread in Figure 21 was made from actomyosin, extracted as such from muscle.

Contraction of actomyosin can be observed even in solutions. If ATP is added to a 0.1% "solution" of actomyosin, containing 0.05 M KCl, the fluid becomes turbid and the substance contracts to a small plug (Fig. 22).

Figs. 21a, b

Fig. 22

Fig. 21. a. Actomyosin thread. b. The same contracted. Magn. 1:30.
Fig. 22. Contraction of an actomyosin solution.

All these reactions are perfectly reversible, as long as secondary cohesions do not disturb the picture. The contracted thread can be brought to relaxation by raising the KCl concentration to 0.25 M, especially in the presence of 0.001 $MgCl_2$ which makes both contraction and relaxation more intense and makes myosin less soluble.

Threads usually contain 98% water and 2% protein and consist, if suspended in 0.05 M KCl, of actomyosin, maximally precipitated by the salt present. On the addition of ATP they usually contract to about 66% of their former length. The thread now consists of 50% protein and 50% water. This is exceedingly close packing; closer packing can hardly be achieved. We may conclude herefrom that the thread consists now of anhydrous material with some entrapped water and that it contracted as far as was structurally possible.

As shown by Fig. 20, the transition from contraction to

dissociation, in the presence of ATP, is very abrupt. In the one tube the actomyosin may be found maximally super-precipitated, in the next completely dissolved and dissociated even if the KCl concentration differs in the two tubes by no more than 15%. The actomyosin is either contracted (super-precipitated) or dissociated, depending on the KCl concentration, with only a very narrow transition zone. Since dissociated actomyosin is not actomyosin at all, we may just as well say that actomyosin in the presence of ATP is stable only in a very narrow range of KCl concentrations and can exist here in the contracted state only. ATP has thus two effects: (1) in a wide range of KCl concentration it prevents the association of actin with myosin or makes actomyosin dissociate; (2) in the narrow range of KCl concentration, where actomyosin is most discharged and is stable, it causes the system to contract.*

The dissociating effect can easily be accounted for. As shown before, ATP has only a charging, hydrating effect on myosin. This charge adds on to the charge already present, whether the latter is due to the incomplete neutralization of the original negative charge of the protein by $K^+$, or whether it is due to the positive charge acquired by the adsorption of excess $K^+$. At a certain charge, the system will dissociate on addition of ATP when its hydration reaches the critical value. This happens, in the presence of 0.1% ATP, in KCl, more dilute than 0.02 M or stronger than 0.16 M, with less than 4 or more than 8 $K^+$ adsorbed. Between 0.02–0.16 M KCl the actomyosin is stable and contracted. It is easy to understand why increase of ATP concentration narrows down further this zone of stability, and why a decrease extends it.

In the experiment reproduced in Fig. 20, the zone of precipitation extended from 0.02 to 0.16 M KCl in the presence of 0.1% ATP. In the presence of 0.01% ATP the zone extended from 0.003–0.3 M KCl, while in the presence of 0.4% ATP it narrowed down to 0.03–0.1 M KCl. The same point is brought out nicely by the experiment reproduced in Table III. One can see the zone of precipitation widening as the ATP is gradually decom-

*Dissociation depends also on the relative concentration of actin and myosin. The more we deviate from the natural 1:2,5 relation, the more easily the complex dissociates.

posed. The border between contraction and dissociation is sharp and one K adsorbed or released brings the dissociated system to contraction and *vice versa.*

TABLE III. *Superprecipitation of Actomyosin at Varied KCl Concentrations*

| | M KCl | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0.100 | 0.106 | 0.112 | 0.118 | 0.124 | 0.130 | 0.136 | 0.142 | 0.148 |
| 1 Min. | X | – | – | – | – | – | – | – | – |
| 2 Min. | XXX | X | – | – | – | – | – | – | – |
| 3 Min. | XXX | XXX | XX | X | – | – | – | – | – |
| 5 Min. | XXX | XXX | XXX | XXX | X | – | – | – | – |
| 15 Min. | XXX | XXX | XXX | XXX | XXX | XXX | XXX | XXX | X |

Parallel to the gradual decomposition of the ATP, the zone of superprecipitation widens. X = superprecipitation.

We thus have no difficulty in explaining the dissociating effect of ATP. The difficulty is with the contraction. Why should ATP which in the case of free myosin caused increase of hydration only, have the opposite effect and cause complete dehydration in the presence of actin? If hydration is a question of charge we must conclude that actomyosin, in this condition, is less charged. The experiments of Banga and Hermann have shown that the affinity of actomyosin for metals and ATP is not materially altered by contraction. Actomyosin while contracting does not thus release any of these substances, and the actual composition of the particles does not change during this process. If there is a decrease in charge, it can result only when the charges of the system balance each other. Contraction, in its essence, must be some sort of an intramolecular or intramicellar rearrangement within the system composed of myosin, actin, ATP, and adsorbed ions in which the charges become better balanced, losing their outward action. Such a rearrangement does not take place in the absence of actin or ATP. Above a certain charge and hydration, actomyosin dissociates, but if this critical charge is not reached and there is no dissociation, the spontaneous rearrangement into this better balanced and more stable structure occurs with the consequent agglutination of particles and dehydration. The observations indicate that the contracted-dehydrated and the dissociated-

hydrated condition are two distinct states, that the particles are either in the one or in the other, and there is no modified state between the two, a conclusion fully borne out by experiments to be discussed later.*

It follows from the reversible nature of the reactions concerned that it does not matter in which order we put the single constituents together. If we mix 0.05 M KCl, ATP, actin, and myosin, the result will be contracted actomyosin, regardless of the order in which we mixed them. This will be the case, at least, if the mixing is done at room temperature or above. If, however, we put these substances together at 0°, there will be no contraction. The high viscosity indicates that actin and myosin have united to form actomyosin, but in spite of the presence of ATP there is no contraction. As we allow the mixture to warm up, it contracts. At room temperature, contraction is complete. This shows that the formation of contracted actomyosin takes place in two steps: (1) the formation of uncontracted-hydrated actomyosin from its elements; and (2) the rearrangement of this system into the dehydrated-contracted structure. This latter is an equilibrium reaction depending on temperature.

Though unable to understand the nature and mechanism of this second reaction, we may study its relations: in the first place, its dependence on concentrations.

At the optimal 0.05 M KCl concentration the rate and intensity of contraction will depend on the ATP concentration. Contraction demands a fairly high ATP concentration. Below 0.01% ATP there is no contraction at all. Comparing this result with the ATP adsorption curves of Banga and Hermann the result suggests that there is no contraction if less ATP is adsorbed than one molecule per unit of 70,000–140,000 g. of myosin. Maximal effect is reached with 0.1% ATP which corresponds to one ATP adsorbed per UW. This is the quantity of ATP adsorbed to myosin *in vivo*. In 0.6 M KCl actomyo-

*The change cannot be explained either by a mutual discharge of actin and the myosin, for there is no stoichiometric relation between actin and myosin. It has been shown by Erdös [33] that actomyosin threads may maximally contract with 7 or 30% actin. Moreover it is probable that actin, in muscle, is isoelectric.

sin is, in the absence of ATP, at the verge of dissociation. Mommaerts [11] has measured the quantity of ATP needed to produce complete dissociation of actomyosin at this KCl concentration and has found that dissociation is complete if there is one molecule of ATP for every 100,000 g. of actomyosin, which suggests that units of MW of 70,000 or 140,000 actually exist in the myosin micel.

Mg added in small concentration (0.001–0.0005 M) considerably narrows down the KCl zone of contraction, promoting dissociation. So, for instance, in the presence of 0.1% ATP, where the zone of contraction extends up to 0.16 M KCl, 0.001 M $MgCl_2$ will bring down the border of contraction and dissociation to 0.1 M KCl. $MgCl_2$ has the same action as increased ATP concentration. As will be remembered, Mg strongly enhances ATP adsorption, thus having the same effect as an increase of the ATP concentration. Mg also makes contraction much more intense and sensitizes actomyosin to the action of KCl. Contraction in 0.01 KCl is very weak and sluggish but if $MgCl_2$ is present in 0.0005–0.001 M concentration (which in itself does not induce contraction) contraction will be most intense. Possibly the increased ATP adsorption is involved here too, but it will be remembered that Mg is also instrumental in linking actin to actin.and actin to myosin.

As found by Gerendás [13] Mn and Co have an action similar to that of Mg. Ca, on the other hand, has an opposite effect, raising somewhat the KCl limit of dissociation* [Erdös, 56]. If Mg and Ca are added simultaneously in equal concentration (0.001 M) Mg acts as if no Ca were present, lowering the limit of dissociation.

Ca, in higher concentration (0.01 M), reversibly inhibits the contraction in KCl. Mg may bring the thread into contraction in spite of the presence of the Ca.

Naturally, all these phenomena depend greatly on the freshness of the myosin preparation. Even a few hours' storage at 0° may make an appreciable difference. The reactions of older preparations are weak and sluggish, and maxima are hifted towards higher ionic concentrations.

*The antagonistic effect of Ca and Mg may be involved in Meltzer's narcosis.

## IV. G-Actomyosin

Like F-actin, G-actin also unites with myosin to form actomyosin, but the union of the two substances is not accompanied by a rise of viscosity, the viscosity of G-actomyosin being equal to that of myosin. That a union has taken place at all is revealed by the fact that the subsequent addition of F-actin does not cause a rise of viscosity, the combining capacity of myosin being satisfied by the G-actin. That G-actin is formed is shown also by precipitating the protein with KCl: the precipitate is more opaque and granular than the precipitate of free myosin.

There is an important difference in the reaction of G- and F-actomyosin with ATP: while F-actomyosin is dissociated by ATP at only high or low salt concentration, G-actomyosin when ATP is added dissociates at any salt concentration. G-actin thus forms no compound with myosin-ATP, regardless of the K saturation. Consequently in the presence of ATP, myosin does not catalyze the G-F transformation of actin.

G-actomyosin forms no elastic gel and is not contractile. If F-actomyosin is brought to contraction by ATP and the contracted actomyosin is rapidly dissolved by adding salt in high concentration (0.6 M) the actin will be found to be present in the globular form, as indicated by the lack of DRF which appears only after time is allowed for the actin to polymerize again. F-actin is thus depolymerized during contraction of actomyosin.

Though G-actomyosin is not contractile, we may prepare contracted G-actomyosin by making F-actomyosin contract. This contracted G-actomyosin is dissociated by ATP, like F-actomyosin, only at high or low KCl concentrations.

## V. A Theory of Contraction

"Super-precipitation" is but an extreme degree of precipitation, "contraction" an extreme degree of shrinking. Both phenomena are merely different expressions for one and the same reaction. Precipitation and shrinking are common occurrences in colloidal chemistry, and are usually due to loss of charge with the consequent agglutination of particles. The immediate cause of decrease in volume is due to the release and expulsion of water. As W. T. Astbury recently pointed out in his Croonian Lecture, the phenomena observed in actomyosin can be classed with colloidal synaeresis. But the rate and extent of these reactions as well as the colloidal and chemical inactivity of ATP are somewhat out of line. The fact that neither actin nor myosin shows similar reactions; that contraction and super-precipitation are specific reactions of the complex of the two materials, a complex built of two widely different and rather peculiar proteins, and representing a rather specific structure; these phenomena suggest that a special mechanism is involved, even though the forces at play may be identical with those generally responsible for analogous phenomena.

In an actomyosin thread the micels have a nearly random orientation and the contraction of the thread is almost isodiametric. The very slight DR indicates a very slight orientation parallel to the axis, and measurement shows that contraction is not perfectly isodiametric.

To quote a measurement of M. Gerendás, a thread which shortened by 62% became thinner by 58%. Gerendás tried to increase the orientation of micels within the thread by stretching. Stretching the thread by 10–15% increased the ratio to 62%:53%. Unfortunately, native actomyosin threads cannot be stretched any more because they break. Gerendás found that the threads can be stretched in glycerol as much as 200%. Threads hereby become doubly refracting and still show some contractility which is distinctly anisodiametric. To quote an example: stretching in 25% glycerol by 200% gave the thread a DR of $15{\cdot}6.10^{-4}$. Contraction became distinctly anisodiametric,

the thread shortening by 16% and becoming thicker by 20%.

Actomyosin threads, denatured by heavy metals, like Cu, are very elastic and can be stretched as much as 300%, but they do not contract any more under the influence of ATP. Gerendás found that lighter metals, like Zn, if applied in high dilution and for short periods, make threads stretchable without completely abolishing their contractility. For instance, a thread, treated with 0.001 M $ZnSO_4$, could be extended by 200%. Its DR, after extension, was $11 \cdot 6.10^{-4}$. On extension the micels became thus oriented parallel to the axis; at the same time a considerable quantity of the intermicellar water was pressed out and appeared in the form of small droplets on the surface. The stretching is elastic but if the thread is kept in a stretched condition for some time it "sets" and does not contract any more if released. Suspended in 0.1 M KCl, the thread contracted on addition of ATP, becoming shorter by 30% and wider by 55%. We thus have a further insight into the mechanism of contraction. If, in the stretched thread, the actomyosin micels run parallel to the axis of the thread, then, evidently, the intermicellar splits run parallel too. But if the intermicellar splits, containing the intermicellar water, run parallel, then the loss of this water could not make the thread shorter, only thinner. Thus the shortening of the thread could not be due to the loss of water but must be due to the shortening of the particles themselves, and contraction of actomyosin cannot be dismissed as a simple case of shrinking due to loss of water. There must be a specific mechanism responsible for the shortening of the micels, a mechanism which explains why the actomyosin particle is built of two specific colloids, why contractility is a specific property of their complex and why these two colloids give off hydrate-water with different degrees of ease. The mechanism would also explain why one of the two colloids is capable of breaking up into globules and does so in every contraction, though a contractile complex is formed by its fibrous form only.

When trying to find a possible answer to these questions we may start from the most striking specific property of actomyosin: the strongly elongated shape of their particles. If we

picture the particle as a double rod, one half of which (actin) is composed of globules, we shall have something like the rod in Fig. 23. If in this system myosin (black) shrinks, or shrinks

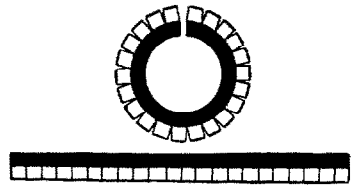


Fig. 23

Fig. 23. Model of relaxed and contracted actomyosin particle.

Fig. 24a

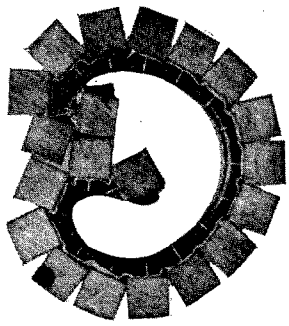


Fig. 24b

Fig. 24. Model of actomyosin micel. The myosin is represented by a rubber tubing which was gently stretched before its ends were fixed to the end of the wood. On release the system curls up. The model was kindly prepared in Prof. Kapitza's laboratory at Moscow.

more than its partner, the system must bend. Geometry demands that if such a system is, for instance, 100 units long and 1 wide, and if one of the partners shrinks by 3 units, then the system should curl up to a complete circle. Such a change would bring

the shrinking partner on the smaller inner circumference. But as the shrinking colloid becomes satisfied by this arrangement, then the other partner must tend to overcome being stretched by being distended on the longer, outer circumference. There will be no trouble, however, if this latter is capable of breaking up into globules, as shown in Fig. 23. The same is shown in Fig. 24. In this model the dehydrating myosin is represented by a rubber tubing which has been stretched gently before attaching it to the ends of the actin-rod. On release of the system, the rubber contracts, imitating the shrinking of myosin. By curling up, the system shortens its actual length by ⅔. The mechanism is an amplifier in which slight changes of volume of two different substances are amplified, thus producing the very extensive motion of shortening.

If the atomic groups linking the actin globules are located on opposite poles of the particle, then bending of the system must disrupt these links even if there is no distention. This is schematically shown in Fig. 25 where the links are symbolized by strips.

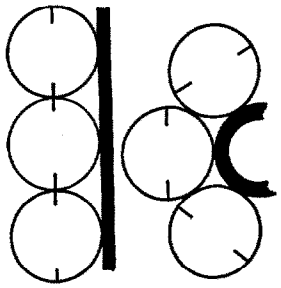


Fig. 25

These models or theories are to be taken with a grain of salt. They are designed to bring out the fact that an elongated system is prone to bend if composed of two parts which change their volume differently. This is nothing new. This simple principle is responsible for the bending of wooden boards or bending of "bimetals." Such a bending, or angular motion, would nicely explain the properties of the elongated actomyosin particle composed of two different colloids.

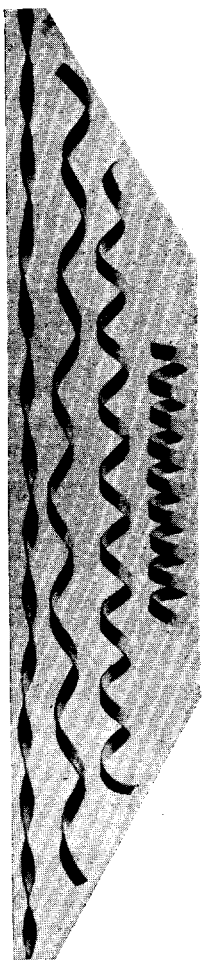
In Figs. 23 and 24, the actomyosin particle was pictured as a system composed of two partners, only one of which shrinks. The anhydrous nature of contracted actomyosin indicates that both proteins lose their hydrophility in the process of contraction. If the change in both is not equal, then, in addition to the general synaeresis, curving, or angular motion also occurs. How far angular motion and how far general synaeresis are responsible for contraction, remains to be shown. Angular motion may clarify considerably the dynamics of the system and explain the specific features of reactions and structure of actomyosin without introducing any new concepts, unknown in colloid chemistry or in everyday life. It also explains why our oriented thread becomes shorter and thicker on contraction and why it loses its DR, as has actually been found by experiment Gerendás 13].

The curling of unequally shrinking double rods can be nicely demonstrated in actomyosin threads, suspended in 0.1 M KCl. If ATP is added to the solution and the fluid is mixed, the ATP reaches the thread from all sides and the thread contracts. If, however, the ATP is allowed to reach the thread from only one side, then this side will contract first and the thread curls up. Ciliary motion possibly has such a mechanism, cilia being built of contractile matter in which waves of contraction pass along on one side, or alternately on the two opposite sides.

On the assumption that the actomyosin forms double rods the bending of which is involved in contraction, the question arises how these actin and myosin rods or threads are actually put together. One can try to approach this problem by constructing models. One will be led to the conclusion that there is only one arrangement which meets all demands: putting the two together in a spiral. Such a spiral may be obtained from the rod in Figs. 23 and 24 by twisting it. On bending, these twisted rods would no longer curl up into a circle but would form something similar to a corkscrew or spiral spring. Such a spiral is symbolized by the model in Fig. 26, in which a flat rod (supposed to consist of a flat actin and myosin half) is twisted. Figs. 27, 28, 29 show the same rod in increasingly bent condition. Fig. 30 shows the effect of different degrees of twist: a′, b′, c′ are the bent structures formed from a, b,

and c. It is evident that *c* will exert the greatest force on contraction, its angular motion being the greatest.

In an earlier paper [70] I tried to explain cross-striation in



Figs. 26-29

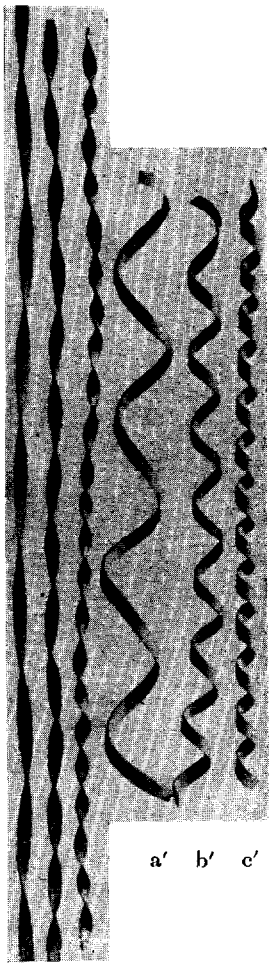



Fig. 30

Figs. 26–29. Model of an actin and myosin rod. Fig. 26 shows the twisted rod; Figs. 27–29 show the same rod in increasingly bent condition.

Fig. 30. Model of an actin and myosin rod, showing the effect of different degrees of twist. a′, b′, and c′ are the bent structures formed from a, b, and c.

muscle by this spiral twist, supposing that one turn corresponded to one period of cross-striation. Evidence obtained since that time on the electron microscope by Hall, Jakus, and Schmitt does not support this contention, and if the theory of the spiral and the angular motion is correct, the twist must be in molecular dimension so that the bent and shortened system, as shown in Fig. 30*c*, may still appear as a straight rod at the magnifications obtainable at present.

However nicely they explain many properties of muscle, spiral structure and angular motion are but theories and there are observations to suggest that the role of actin is not a geometrical one. MW measurements suggest that in muscle one actin particle unites with each sub-unit of the myosin micel. If this is the case in muscle then in "B-myosin" there is one actin particle for every two myosin sub-units. Erdös found that actomyosin shows maximum contractility only when its actin content is at least 8%. At this limit there would be one actin particle for four myosin sub-units (four being the greatest number of cubic particles which can be simultaneously in touch with one globular particle on the surface of their system). These relations are difficult to reconcile with a geometrical function of actin and rather suggest a direct chemical influence of actin on myosin.

## VI. Energy Changes in Contraction

The observations discussed in the previous chapters suggested that the contracted and uncontracted conditions of actomyosin represent two distinct states of this matter, and that the transition from one to the other is an equilibrium reaction, dependent on temperature. The free energy change of an equilibrium reaction can be calculated from its equilibrium constant ($\Delta F = -RT \ln K$) while the heat of the reaction can be calculated from the dependence of the equilibrium constant on temperature by means of van't Hoff's equation:

$$\frac{dlnK}{dT} = \frac{W}{RT^2}$$

which, if integrated between the temperatures $T_1$ and $T_2$, gives

$$2.303(\log K_2 - \log K_1) = -\frac{W(T_2 - T_1)}{R\ T_2 T_1}$$

where $W$ = heat of reaction, $R$ = gas constant 1,986 cal., $T$ = abs. temp. The problem is to find the equilibrium constant of the reaction (contraction) at different temperatures. If contraction and relaxation are two distinct states and the single micels are either fully relaxed or maximally contracted, we can draw conclusions about the equilibrium from the length of the thread. Maximal contraction in this case means that 100% of the micels are contracted, whereas no contraction means 0% of the micels are contracted. If the thread shortens maximally from 100 to 30, then this 70% contraction actually means that 100% of the micels contracted. Contraction half-way from 100 to 65 would mean that 50% of the micels contracted producing the equilibrium 1/1. Thus the length of the thread could give direct information about the equilibrium constant at any temperature; and in order to find this constant the length of the thread would simply have to be measured after the thread reached its equilibrium length, provided one had ideal threads of constant composition and orientation.

L. Varga considered the muscle fibre to be a similar perfect actomyosin thread and used as experimental material the psoas

of the rabbit, which is built of parallel-running fibres. He washed out the muscle *in situ* with ice water and cut it into slices on the freezing-microtome in such a way that each slice contained one sheet of parallel-running fibres. After the length was

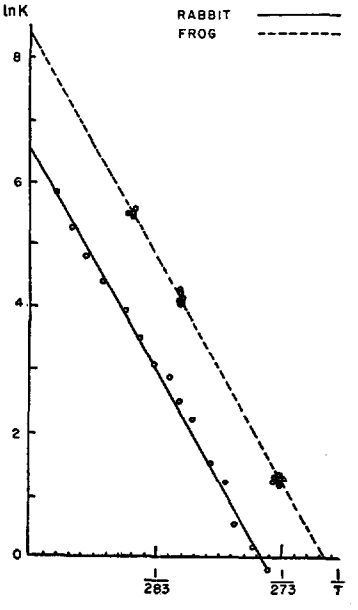



Fig. 31

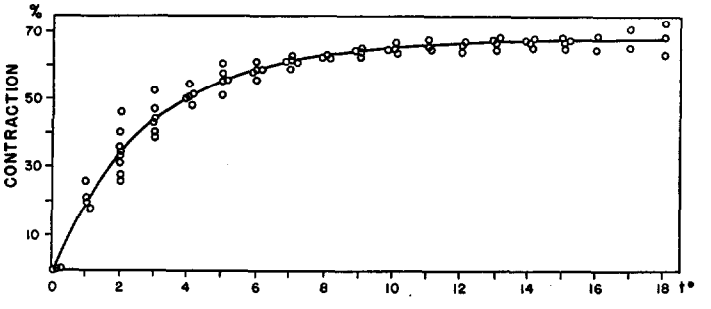



Fig. 32. Contraction of muscle slices at varied temperature in presence of KCl and ATP.

measured, the slice was transferred into a KCl-ATP solution of known temperature and its length measured again after maximal contraction was reached. The natural logarithm of

the equilibrium constants plotted against 1/T gave a straight line (Fig. 31) which proved both of Varga's suppositions to be correct: that muscle actually behaves as an ideal thread, that contraction and relaxation are distinct states; and that an actomyosin micel is either fully relaxed or fully contracted, there being no transitional states. If the muscle contracts only half-way it is not because the micels are half-contracted, but because only half of the micels are in the contracted state.

Figure 32 shows the result of the actual measurements. At 0° there is no contraction. With rising temperature the contraction becomes rapidly stronger to reach a maximum of

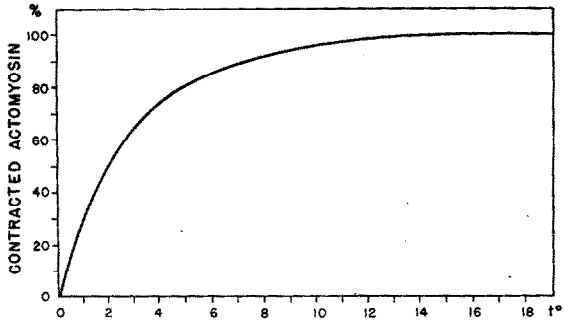



Fig. 33. Same curve as in Fig. 32, maximal (70%) contraction being marked as 100%.

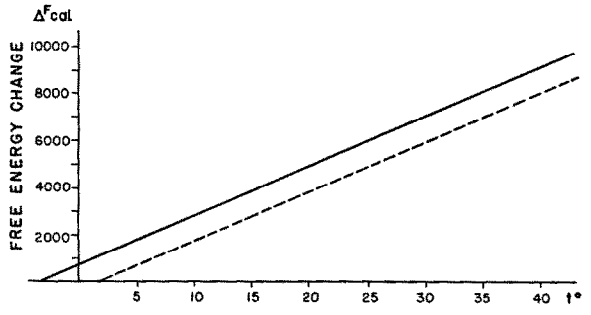



Fig. 34. Free energy change in muscle slices on contraction at varied temperatures. Upper curve: frog; lower (broken) curve: rabbit.

68–70% asymptotically at about 16°. Figure 33 is the same curve, the 70% maximal contraction being marked as 100% contraction. This 100% means that 100% of the micels are in

the contracted state: 50 on the ordinate means that the muscle is half-contracted and that 50% of the micels are in the contracted state. From this curve the equilibrium constants for different temperatures can be read. The equilibrium constants $K = \frac{\text{contracted actomyosin}}{\text{relaxed actomyosin}}$ are given in Table IV. The $\triangle F$ for different temperatures is given in the lower (broken) curve of Fig. 34. As can be seen, the $\triangle F$ decreases with decreasing temperature to cut the abscissa about 2°, which explains fully why muscle does not contract at 0°. The heat of the reaction,

TABLE IV

| t° | T° | Contract. in %, max (68) = 100 | K. | Log. K. | Ln. K. |
|---|---|---|---|---|---|
| 1 | 274 | 30.66 | 0.43 | 0.634—1 | — |
| 2 | 275 | 51.00 | 1.04 | 0.017 | 0.039 |
| 3 | 276 | 64.00 | 1.69 | 0.230 | 0.53 |
| 4 | 277 | 75.00 | 3.00 | 0.477 | 1.09 |
| 5 | 278 | 82.00 | 4.55 | 0.658 | 1.52 |
| 6 | 279 | 86.10 | 6.19 | 0.792 | 1.82 |
| 7 | 280 | 88.99 | 8.07 | 0.907 | 2.09 |
| 8 | 281 | 92.00 | 11.05 | 1.060 | 2.45 |
| 9 | 282 | 93.72 | 14.79 | 1.170 | 2.69 |
| 10 | 283 | 95.73 | 22.40 | 1.351 | 3.12 |
| 11 | 284 | 96.50 | 30.55 | 1.485 | 3.42 |
| 12 | 285 | 97.44 | 38.11 | 1.581 | 3.65 |
| 13 | 286 | 98.19 | 54.33 | 1.735 | 4.00 |
| 14 | 287 | 98.63 | 72.11 | 1.858 | 4.28 |
| 15 | 288 | 98.99 | 98.86 | 1.995 | 4.60 |
| 16 | 289 | 99.40 | 165.00 | 2.218 | 5.05 |

calculated from these data, is −56,000 cal. Contraction is thus an endothermic process. At the same time the free energy drops at 37° by 7,000 cal. At 0° there is no drop of free energy and for this reason rabbit muscle does not contract at 0°.

It was rather shocking to find a frog swimming about in ice water after the conclusion of these experiments, and we had to decide whether the frog or Mr. Varga was wrong. The experiment was therefore repeated using a slightly modified technique with frog muscle. The heat of the reaction was found to be the same as in the rabbit experiment (53,500 cal.) but

the free-energy change was greater, 8,500 cal. as compared to the 7,000 cal. in the rabbit experiment (37°). In Fig. 34 the $\triangle F$ of the rabbit and frog are compared: the lines are parallel but in the frog there is still a decrease of the F at 0° which enables him to swim in ice water as he sometimes must do. Thus $-3°$, where his $\triangle F$ is 0, no longer interests him because at this temperature he is frozen anyway.

Varga's experiments allow different conclusions. Since the free energy decreases in contraction, we can postulate that this process, being a transition from an energy-rich relaxed state to a stabler state, poorer in free energy, occurs spontaneously. Relaxation however can occur only if the free energy of the system is increased by 7,000 cal. (if the process is carried out isothermally, as is true in the case of the rabbit). The splitting of ATP which, according to our present knowledge, is the source of muscular energy, is thus needed for relaxation. The free energy of ATP is unknown; the breaking of one phosphate link liberates 11,000 cal. Provided that this energy change is not very different from the $\triangle F$, then the breaking of one such link is enough to relax one actomyosin particle.

ATP thus has two functions. One is a static function, independent of splitting. ATP acts as a building stone of the contractile system. Without ATP, the actomyosin does not contract or relax: it is not reactive at all. The other function of ATP is to provide energy for relaxation.

## VII. Enzymatic Functions of Myosin and Actomyosin

### *ATP-ase and ADP-ase Activity. Dephosphorylation and Deamination. Protein II.*

The most important discovery in this field is that of Engelhardt and Ljubimowa who showed that it is myosin itself which splits off the first phosphate of ATP. This discovery was corroborated in many quarters (D. M. Needham, K. Bailey 1942) and was borne out by the very wide experience of my own laboratory with crystalline myosin. The enzymatic activity of myosin is not altered by repeated recrystallization.

According to Engelhardt and Ljubimova, only the first phosphate is attacked by myosin while the second phosphate is split off by a different, water-soluble enzyme. This latter view is undoubtedly erroneous and there can be little doubt that the second phosphate too is split off by the myosin itself, but this reaction is effected only in the presence of a water-soluble and acid-stable protein.

Kalckar was the first to describe an acid-stable protein involved in the splitting of the second phosphate. He called it "myokinase" and attributed to it the function of a dismutase which dismutates two ADP molecules into one molecule of AMP and one molecule of ATP. The ATP is dephosphorylated again to ADP and so the reaction goes on until all the ATP is dephosphorylated to AMP.

Independently of Kalckar's observation, Laki [26] also showed that an acid-stable, water-soluble protein is involved in the splitting of the second phosphate of ATP by the insoluble muscle-residue. The importance of this reaction was strongly enhanced by Laki's experiments which showed that the splitting of the first and the second phosphate had different pH optima, the former being at pH 7.4, the second at 8.6. This suggested that the splitting of the first and second phosphate were independent enzymatic functions of myosin and that there is no dismutation. If we assume dismutation occurs, then it is only ATP that is split.

Banga, taking up the work with crystalline myosin, found that this substance rapidly splits off one P from ATP but is capable of dephosphorylating the ADP formed only if acted on first by a soluble protein to which she attributed the function of an isomerase. Since no ATP or AMP was found, there could have been no dismutation.

In her later, extensive studies, pursued partly in collaboration with Josepovits, Banga was led to a different conception. She found that the water-soluble and acid-stable protein which she called "Protein II" did not act on ADP directly at all; it acted only in conjunction with myosin, completing and modifying the activity of this substance.

Whether Banga's protein II and Kalckar's dismutase were identical is difficult to say. It is possible that there is a whole group of water-soluble and acid-stable substances in muscle, acting with myosin.

These experiments of Banga furnished no conclusion about the chemical mechanism of the dephosphorylation of ADP. They simply showed that the second phosphate is split off only in the presence of myosin *and* protein II. At the same time they also showed that simultaneously with the second P, N is also detached and the ADP is deaminated. This reaction too takes place only in the presence of protein II *and* myosin. Efforts to fractionate protein II into two substances, one acting on dephosphorylation, the other on deamination, failed. The experiments thus indicate that ADP is deaminated under the combined action of myosin and protein II. Deamination occurs only after the first P is split off. Deamination and the splitting of the second P run so closely parallel that it is highly probable they do not represent different enzymatic functions, but are the result of one reaction. In this reaction, one of the phosphate groups in ADP is attached to the $NH_2$ group and is detached together with this $NH_2$ group. This ADP, with one phosphate attached to the $NH_2$ group, was called $ADP_2$. Its existence was supported by Banga's isolation of an ADP with its $NH_2$ group masked.

According to Banga, the ATP prepared from muscle and used in our experiments has the Lohmann formula, i.e., none of

its phosphates is attached to the amino group, which is free. The three phosphates are attached to the carbohydrate end of the molecule. If one phosphate is split off, an ADP results which has its two P's in its original position. This ADP is called by Banga "$ADP_1$." According to the observations of Banga, it is most probable that by the system myosin + protein II, one of the phosphates of $ADP_1$ is transferred to the amino group, whereby $ADP_2$ is formed. It is then this phosphorylated amino group which is split off by myosin + protein II. At the moment we must stress observations rather than comments. The observations could be equally well explained by supposing that protein II is an enzyme which transfers the phosphate in ADP to the amino group. We should have to suppose only that the equilibrium point of this reaction is rather on the side of $ADP_1$ and the concentration of $ADP_2$ is too low to be detected at present by direct chemical methods, but not too low to allow a rapid deamination of the phosphorylated amino group.

These results of Banga make it highly probable that deamination of ADP is a phospholytic reaction, that the $NH_2$ group is phosphorylated prior to its detachment. Such a transfer of phosphate from the carbohydrate to the $NH_2$ group seems to occur in ADP only, after the splitting off of the first P of ATP. These results settle the old problem of whether ADP is deaminated or dephosphorylated first: P and N go off together. Both the first and second phosphate of ATP are split off by myosin, but the second is attacked only after there has been a P transfer and the amino group has been phosphorylated. Whether the P transfer is an independent function of protein II and the P splitting an independent function of myosin, or whether myosin and protein II perform both functions together is not known.

If crystalline myosin acts on ATP in the absence of protein II the reaction stops after the first P has been split off. The products of this reaction have been analyzed by Banga and Josepovits [55]. Along with $ADP_1$ a dinucleotide was found, composed of one molecule of ATP and one molecule of ADP. It contained five P but had only one free $NH_2$ group. The simplest explanation is that there was some $ADP_2$ formed during the dephosphorylation which was dimerized with ATP,

or else that $ADP_1$ was formed and dimerized with ATP in such a way that the phosphate of the one molecule was linked to the $NH_2$ of the other. As shown before, if protein II is added to this mixture of $ADP_1$, dinucleotide, and myosin, the reaction proceeds and the second P is split off together with the N. One would be inclined thus to suppose that these substances, $ADP_1$ and dinucleotide, represent steps in the normal splitting of ATP. This, however, is definitely not the case, because the splitting of $ADP_1$ and dinucleotide is rather slow, and the splitting of dinucleotide even has a considerable latent period. But the action of crystalline myosin on ATP in the presence of protein II is rapid, and the phospholytic deamination follows the first dephosphorylation at a high speed without a latent period, ATP being split into inosinic acid, phosphate, and $NH_4OH$. Thus $ADP_1$ and dinucleotide are stabilization products and not normal intermediates. The normal primary product of the first dephosphorylation of ATP (which is formed if this dephosphorylation takes place in the presence of protein II) is a different substance which is attacked at once.

The presence of actin makes no difference if myosin and protein II are made to act on ATP. If, however, myosin and protein II are made to act on $ADP_1$ or dinucleotide, actin greatly speeds up the reaction and there is no latent period. In this case the system is thus fully active only if three proteins work together: myosin, protein (hence the name "protein II"), and actin. While myosin splits $ADP_1$ and dinucleotide slowly — the latter with a latent period — actomyosin, in the presence of protein II, attacks these substances readily.

### *Metals: Physical State and Enzymatic Activity*

As Fig. 35 taken from the early work of Banga [10] shows, metals have a decisive influence on the enzymatic activity of actomyosin. All metals that do not denature protein have an activating effect. There is a distinct maximum of activity, both metal-free myosin and myosin with too much metal being inactive. The activation is of the same order with most metals, only Ca and Mg having a specially strong action.

Figure 36 shows the effect of varied KCl concentration on

the phosphatase activity of pure myosin and actomyosin. The very small enzymatic activity at the lowest salt concentration

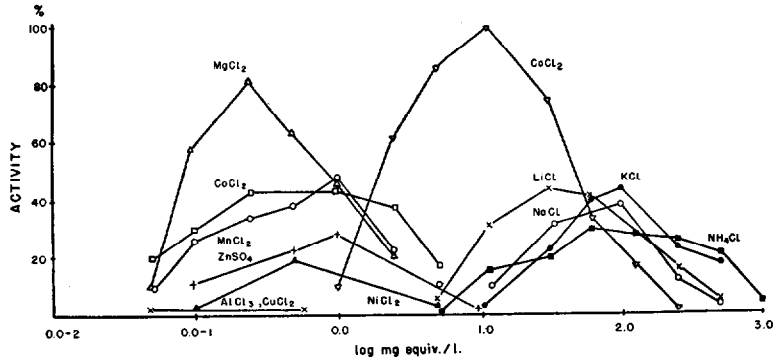



Fig. 35. Effect of various salts on the phosphatase activity of natural, impure actomyosin. 100% activity: maximal effect observed.

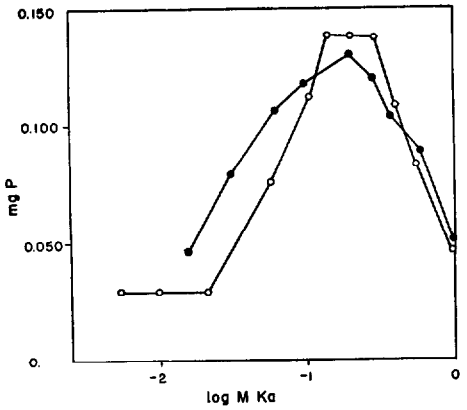



Fig. 36. Effect of KCl on the phosphatase activity of myosin and actomyosin. Ordinate: mg. P liberated. Incubation for 5 min. at 38°. Points: myosin. Circles: actomyosin [Banga and Szent-Györgyi, 36].

is probably due to the K introduced as cation of the ATP used as substrate. As can be seen, there is no real difference between myosin and actomyosin. Whether the two are really identical we do not know because in all these enzymatic experiments ATP has to be present as the substrate. In the presence of ATP, however, the actomyosin dissociates at the higher and lower

salt concentrations; hence at these concentrations both curves are but curves of myosin. The curves do not show any evident relation between the enzymatic activity of myosin and its physical state.

Ca very strongly activates both myosin and actomyosin. The highest enzymatic activities were observed in the presence of this metal. According to Banga's latest results Ca also strongly activates all ADP-ase activities in which protein II is involved.

A very unexpected effect is the activity of Mg. This metal (0.01–0.001 M) strongly enhances the ATP-ase action of actomyosin — being in this respect but little inferior to Ca — but very strongly inhibits the same activity of myosin: 0.001 M Mg is sufficient to cause almost complete inhibition. In the presence of Mg, actin thus has decisive influence on the activity of myosin, turning a strong inhibition into a strong activation.

This strong inhibitory action of Mg on myosin makes it possible to study the action of its varied concentrations on the enzymatic activity of actomyosin. In the case of other metals, for instance KCl, this was not possible because at higher or lower salt concentration the actomyosin dissociated, the free

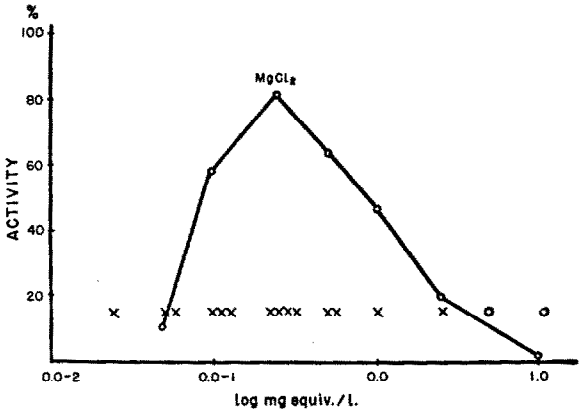



Fig. 37. Effect of varied concentrations of $MgCl_2$ on the phosphatase activity and physical state of actomyosin. Superprecipitation is noted with x 100% activity, maximum activity of the same preparation (obtained with Ca).

myosin took over the enzymatic activity, and it was impossible to say what was due to myosin and what to actomyosin. In the case of Mg the situation is different because the myosin formed on dissociation is inactive in the presence of Mg. So the ATP-ase action observed can only be action of the actomyosin.

Figure 37 shows the influence of varied concentration of Mg on the ATP-ase action of actomyosin from the early studies of Banga on impure, natural actomyosin. In a parallel experiment the physical state of the actomyosin was observed. Super-precipitation (contraction) was marked on the curve with crosses. As the curve shows, there is a complete parallel between super-precipitation and enzymatic activity. Where there is maximal contraction, there is maximal enzymatic activity; where there is partial or no contraction, there is partial or no enzymatic activity. But we know from the experiments of Varga that at the temperature employed (37°) the actomyosin particles could be either contracted or dissociated, and partial contraction when present does not result when the micels are partially contracted but when only part of the micels is contracted. The same must hold true for the enzymatic activity: partial enzymatic activity means that only part of the micels is active. Therefore we must conclude that only the contracted micels are enzymatically active. (This must necessarily be so since if they are not contracted they are dissociated and thus inactive.)

The myosin micel splits ATP thus in its contracted, dehydrated and energy-poor condition only, when it needs energy for its relaxation. As soon as it has liberated the energy necessary for its relaxation and has actually relaxed, it goes over into its enzymatically inactive, hydrated, relaxed, and dissociated state.

Since muscle contains Mg in rather high concentration, these observations seem to give the clue to one of the most basic biological problems: that of the regulation of energy liberation. In our system it is the need for energy which entails its liberation. The system is built in such a way that, in the presence of Mg, it is active only in its energy-poor state, when it needs energy in order to return to its energy-rich state, the state of rest. As soon as it has regained energy, splitting ceases, since the protein in this modification is enzymatically inactive.

It follows from the above data that KCl also must inhibit the ATP-ase action of actomyosin if added to the system in the presence of Mg, in a concentration sufficiently high to induce dissociation. While 0.001 M Mg and 0.2 M KCl both strongly promote enzymatic activity of actomyosin, together they will produce complete inhibition. The action, though striking, can be explained easily. This is not the case with the unexpected reaction obtained if Ca and Mg are added simultaneously to actomyosin. Both $CaCl_2$ and $MgCl_2$ strongly promote ATP-ase activity of actomyosin. One would expect a summation of this effect if both are added simultaneously. Instead, a very strong inhibition is observed which is almost complete if the two metals are added in 0.01 M concentrations, though when added one by one they produce maximal activation. It is difficult to explain this interaction. Possibly the Ca neutralizes the influence of actin on myosin and the myosin, released from the influence of actin, is now inhibited by the Mg. Whatever the explanation may be, it is possible that these subtle ionic synergisms and antagonisms may play an important role in the biological functions and regulations of the system. An ion-exchange between actin and myosin may completely change the reactions. The ATP-ase action and its relation to ions is a fascinating problem. Work here is facilitated by the great stability of phosphatase action of myosin which may be undiminished even after a fortnight's storage at 0°.

## VIII. ADP Contraction, Protein I, and Fluochrome

If crystalline myosin is washed carefully and is allowed to act upon ATP, the ATP is dephosphorylated and the products of its splitting are stabilized in the form of $ADP_1$ and an ATP-ADP dinucleotide, substances which are no longer acted upon by myosin.

If pure myosin is mixed with actin to form actomyosin, this actomyosin will readily contract under the influence of ATP but not on addition of the mixture of $ADP_1$ and dinucleotide. Contraction occurs, as shown by Guba, if a small amount of a watery muscle extract is added. The substance, present in the aqueous extract and responsible for this action, is an acid-stable, thermolabile protein, which will be called here "protein I." In impure condition the substance can be boiled in 0.1 N HCl for 15 minutes without loss of activity. At neutral reaction it stands short boiling only. The purified substance at room temperature is not inactivated at pH 1 but is destroyed in ten minutes at neutral reaction at 55°. The substance can be precipitated by trichloracetic acid and redissolved by neutralization in active condition, though this treatment makes it rather labile. The IP of the protein is pH 4.6. It precipitates below pH 1.

The activity of the protein was expressed in units, one unit being that quantity which when added to 2 ml. fluid (containing 2 mg. actomyosin, 1.2 mg. ADP in 0.05 M KCl, and 0.001 M $MgCl_2$ at pH 7) caused contraction within 1–2 minutes at 22° C. The protein was purified in the following way: the meat of the freshly killed rabbit was minced and suspended in an equal weight of water and allowed to stand overnight at 0° C., then filtered through a cloth, pressed out, the juice centrifuged. The liquid was brought to pH 1 by means of concentrated HCl and neutralized with KOH. The heavy precipitate, formed on neutralization, was eliminated on the centrifuge, the fluid cooled to 0° and mixed with an equal volume of acetone at −15°. The mixture was centrifuged at once at 0°. The active substance was in the precipitate. The acetone was eliminated and the residue dissolved in water.

The insoluble fraction was separated on the centrifuge and rejected. The solution was then saturated to 0.5 with ammonium sulphate at 0°, the precipitate eliminated by centrifugation, the saturation increased (at 0°) to 0.7, and the precipitate eliminated again. Then the fluid was brought to pH

4.6 (at 0°) and the precipitate, which contained the active principle, was separated on the centrifuge.

In the course of this preparation half of the active substance was lost. While the primary muscle-extract contained 40 units and 30 mg. protein per ml., the end product contained one unit for 10–20 γ protein which corresponds to an average of 50 x purification.

If an active extract is added to the actomyosin-ADP mixture, there is no action for 10–120 seconds, and then contraction sets in. The length of time necessary to produce

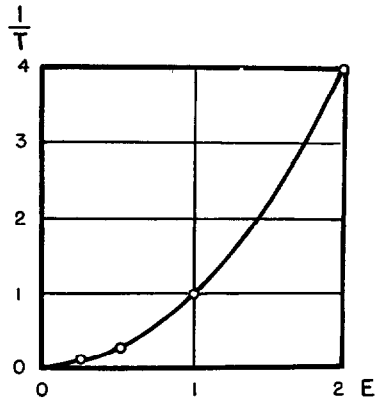



Fig. 38. Relation of number of units (E) of protein I to the reciprocal value of time necessary to produce contraction.

contraction depends on the activity of the extract (E). The relation between the two quantities is given in Fig. 38. The curve can be expressed by the equation $1/T = K.E^2$.

If the purified extract is brought to pH 9–10, within a few hours it turns yellow and loses its activity. If cysteine is added, the color disappears and the activity returns. Evidently the active substance is a chromoproteid with an auto-oxidizable prosthetic group which is active in its reduced state only. In the UV the substance shows a splendid fluorescence which disappears on reduction but not on acidification. This fluorescence is distinctly more bluish than that of the yellow enzyme or lactoflavin.

The chromatogram of the reversibly oxidized active substance, measured by means of the chromatograph of König-

Martens, is given in Fig. 30. As will be seen, the maximum, being at 460 m$\mu$, is somewhat shifted towards the higher wavelength as compared to the chromatogram of lactoflavin, but the main difference is an absorption above 500 m$\mu$ not given by lactoflavin.

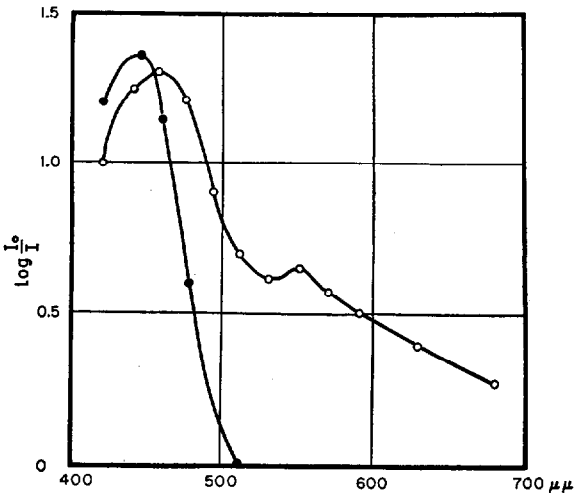



Fig. 39. Chromatogram of oxidized protein I (circles), as compared to the curve of lactoflavin (points).

The dye can be detached from the protein by dialysis at alkaline reaction or by treatment with 20–50% acetone. The detached dye is labile and, at alkaline reaction, readily goes over into a reddish-brown substance which no longer fluoresces. Possibly, the oxidation product adds to the extinction above 500. This irreversible oxidation occurs also in the chromoproteid on prolonged storage at alkaline reaction. The chemical behavior of the dye makes it probable that it is linked to a nucleotide.

The above data make it clear that the dye can be identical neither with lactoflavin nor with thiochrome. It seems likely that it has vitamin activity and is involved in muscular pathology.

If the ADP is incubated with protein I, the protein eliminated, and the ADP allowed to act on actomyosin, no contrac-

tion occurs, the situation being analogous to that with protein II of Banga. Though the reactions of both substances towards precipitating agents, as well as their lability, are very similar, they seem not to be identical, since Banga's protein II, if inactivated by alkali, cannot be reactivated by cysteine. Guba's purified extracts were always active in Banga's enzymatic test. Activity was lost in both tests by oxidation. If reduced again, activity returned in Guba's test but not in Banga's. Whether the protein I has any relation to Kalckar's myokinase, cannot be stated.